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The resistance of cancer cells to multiple chemotherapeutic agents is a major impediment in the successful treatment of breast cancer. Our results demonstrate that development of multidrug resistance in a human breast cancer cell line (MCF-7) is a result of propagation one or more inherently resistant subclones. One distinct feature associated with the development of multidrug resistance phenotype in breast cancer was that these cells expressed inordinately high levels of a protein cross-linking enzyme, tissue transglutaminase (tTGase). tTGase is a multifunctional protein that in addition to catalyzing protein cross-linking reactions, can bind and hydrolyze GTP and serve as an integrin-binding co-receptor for fibronectin; the features that endows tTGase the properties of a signal transduction molecule. We believe that tTGase expression can confer multidrug resistance phenotype to cancer cells by promoting integrin-mediated cell-survival and cell growth signaling pathways.

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INTRODUCTION

Chemotherapy with cytotoxic drugs such as doxorubicin has been the most common and effective method for treating patients with advanced cancer. Even though this therapeutic paradigm has dramatically improved response rate and prolonged patient survival, the treatment ultimately fails in many cases due to the emergence of cancer cells that no longer respond to anticancer agents. These cells are often characterized by their simultaneous cross-resistance to several structurally and functionally independent drugs. This phenomenon, known as multidrug resistance (MDR), constitutes the major impediment in successful treatment of breast cancer. The cancer cell lines selected for drug resistance *in vitro,* have served as excellent models in understanding the mechanisms that may contribute to the development of MDR phenotype. Although many biochemical mechanisms responsible for the development of MDR phenotype have been defined[1-3], only few have been confirmed in the clinic, and translational studies have frequently proved disappointing[4,5]. Precise understanding of molecular mechanisms that contribute towards the development of drug resistance is imperative to develop strategies for treating such aggressive forms of tumors. Delineation of the molecular alterations that are associated with acquired drug resistance may provide useful leads in this direction.

Based on a previous report that several anticancer drugs, including doxorubicin could serve as high affinity substrates in tissue transglutaminase (tTGase)-catalyzed cross-linking reactions[6] and our own observations that tTGase expression is substantially increased in human breast carcinoma (MCF-7/DOX) cells selected for resistance against doxorubicin[7], we proposed to determine the role of tTGase in acquired resistance to doxorubicin in MCF-7/DOX cells. The following specific aims were proposed to test this hypothesis:

1. To investigate the ability of tTGase from drug-resistant breast tumor cells (MCF-7/DOX) to utilize doxorubicin as an -acyl acceptor substrate.
2. To determine the effect of tTGase-catalyzed cross-linking of doxorubicin on its efficacy to kill breast tumor cell lines.
3. To investigate the role of tTGase in acquired resistance to doxorubicin.

Drug-sensitive MCF-7 cells (MCF-7/WT) will be transfected with full-length tTGase construct while MCF –7/DOX cells will be transfected with antisense tTGase construct. Stably transfected cells will be selected and screened for tTGase expression and tested for doxorubicin-induced toxicity.

BODY

We initially determined whether elevated level of tTGase expression is an isolated phenomenon associated with the development of drug resistance in MCF-7 cells, or it represented a general phenomenon in other cancer cells as well. We tested several pairs of cell lines selected for resistance against MDR-related drugs. Interestingly, regardless of their origin or source, the cancer cells when selected for resistance against MDR-related drugs exhibited increased tTGase activity. The increased enzyme activity in drug-resistant cells was associated with a parallel increase in tTGase protein levels, as established by Western blot analysis[8] (See the appendix for reprint by Chen, Aggarwal & Mehta, 2002). Next, we determined the direct involvement of tTGase in the acquired drug resistance. Since numerous anticancer drugs including doxorubicin were reported to serve as substrates for tTGase – catalyzed cross-linking reaction[6]. We sought to determine whether tTGase could mediate drug resistance by cross-linking and neutralizing the doxorubicin. The ability of doxorubicin to compete against [^{14}C]-putrescine in tTGase-catalyzed reaction was determined. The presence of doxorubicin did not affect the incorporation of putrescine into dimethylcasein. Moreover, radiolabeled doxorubicin failed to serve as an amine donor substrate for tTGase in an in vitro enzymatic assay. These observations thus ruled out the possibility that tTGase can mediate drug-resistance by its ability to utilize doxorubicin as a substrate.

Effect of forced tTGase expression on doxorubicin sensitivity in MCF-7/WT cells.

To directly assess the role of tTGase in drug-resistance, we transfected the drug-sensitive MCF-7 cells with tTGase cDNA. Following the transfection, a transient expression of tTGase protein was observed in MCF-7 cells (Fig. 1A) but the clones expressing tTGase failed to survive. After 1 or 2 passages the tTGase positive clones died, probably by apoptosis (Fig. 1D). Under similar conditions, however, several clones expressing tTGase cDNA in which a cysteine residue in the active site had been replaced with serine (Fig. 1B) were successfully isolated (Fig. 1C). These results suggested that like many other cell types[9], MCF-7 cells are unable to sustain the presence of catalytically active tTGase.

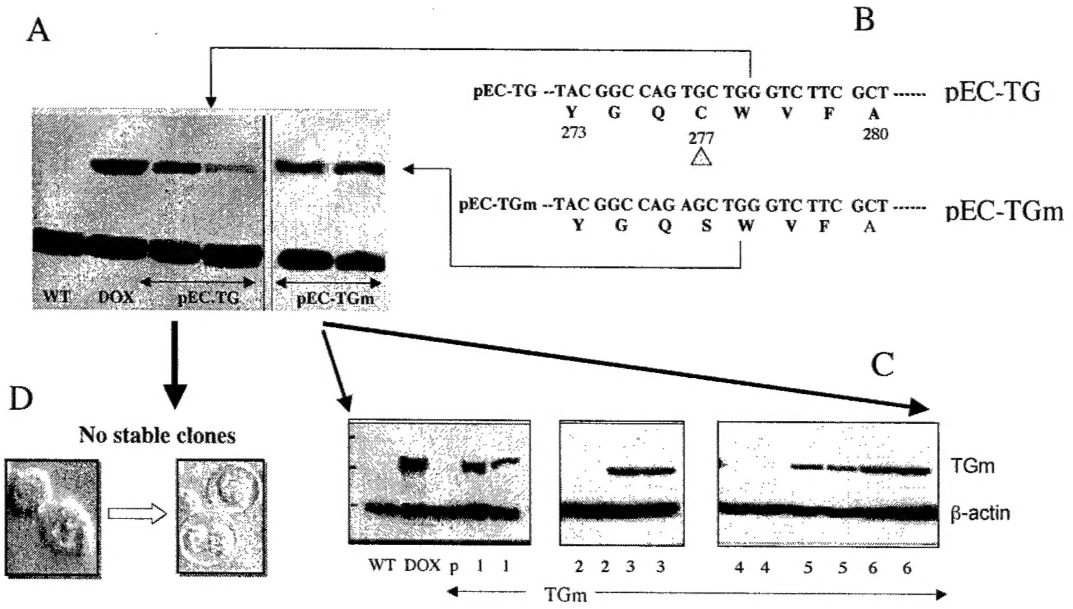

Figure 1: Transfection and selection of MCF-7 cells expressing tTGase. MCF-7 cells were transfected with 1.1 kb cDNA, coding for catalytically active tTGase (pEC.TG) or inactive mutant tTGase (pEC.TGm) (A), in which the active site cysteine (C277) has been replaced with a serine (B). Stable clones expressing only the TGm were selected (C); the MCF-7 clones expressing wild-type tTGase consistently died after 1 or 2 cycles (D). WT, drug-sensitive MCF-7 cells; DOX, MCF-7 cells selected for resistance against doxorubicin; p, MCF-7 cells transfected with plasmid alone.

We then used one of these mutant-TGase transfectd clones (MCF-7/TGm3) to determine the sensitivity of MCF-7 cells against doxorubicin-induced cytotoxicity in a MTT assay. Interestingly, when MCF-7/TGm cells were cultured on uncoated plates, doxorubicin-induced killing was identical to those observed in plasmid-alone transfected MCF-7 control cells. However, under identical conditions, except that the cells were now cultured on fibronectin-coated plates (BD Biosciences), the TGm3 cells showed 2-2.5-fold higher resistance to doxorubicin-induced cytotoxicity as compared to the parental MCF-7 cells. A similar effect of fibronectin and collagen-coated surfaces on cell survival in response to paclitaxel was recently reported for MDA-MB-231 cells[10]. Indeed, when we examined the MDA-MB-231 cells, they showed high basal levels of tTGase expression. We, therefore, conclude that tTGase may endow a drug-resistance phenotype to breast cancer cells by up regulating the anti-apoptotic and cell survival signaling pathways. Indeed, tTGase has been shown to interact directly with multiple members of β1 and β3 integrin family of proteins[11-13]. It is likely that tTGase

augments the interaction of integrins with extracellular matrix proteins and thereby renders the cells chemoresistant by up regulating integrin-mediated cell-survival and anti-apoptotic signaling pathways. In a recent study, Han and Park[14] observed that doxorubicin-resistant lung cancer cells (PC-14/ADR) contained about 10-fold higher tTGase than the drug-sensitive PC-14 cells and that reduction in tTGase expression by stable transfection with the tTGase-specific antisense or ribozyme, rendered the PC-14/ADR cells sensitive not only to MDR-related drugs (doxorubicin or vincristin) but also to non-MDR related drugs (cisplatin). In our own hands, the inhibition of tTGase by transfection with tTGase cDNA in an antisense orientation resulted in spontaneous apoptosis of MCF-7/DOX cells. These observations suggest that tTGase may modulate cells' response to drug-induced apoptosis by up regulating the integrin-mediated cell survival and anti-apoptotic pathways.

If the MCF-7 cells fail to sustain the expression of tTGase (Fig. 1A); how could the drug-resistant (MCF-7/DOX) cells tolerate and survive the expression of this protein? We examined several possibilities to address this dogma and concluded that deficient/defective intracellular calcium pools in MCF-7/DOX cells may account for their successful survival in the presence of tTGase. The deficient intracellular calcium pools can render tTGase catalytically inactive and make the cells permissive to its expression. In agreement with this consideration, elevation of cytosolic calcium by treatment with calcium ionophore, A23187, induced potent apoptotic response (>70%) in $tTGase^+$ MCF-7/DOX cells as compared $tTGase^-$ MCF-7 cells (20-25%)[15]. In addition, the MCF-7/DOX cells also showed higher sensitivity to staurosporine-induced apoptosis as compared to the drug-sensitive MCF-7 cells[16].

Key Research Accomplishments

Our results demonstrate that:

1. Regardless of their origin and source, the cancer cells when selected for resistance against MDR-related drugs, exhibit increased levels of tTGase expression.
2. Drug-resistant MCF-7 (MCF-7/DOX) cells exhibit deficient/defective intracellular calcium pools (see the reprint by Chen, Aggarwal & Mehta in the appendix) that permits them to sustain the expression of tTGase.
3. Activation of tTGase by elevating intracellular calcium levels induces potent apoptotic response in MCF-7/DOX cells (ref. 15).
4. MCF-7/DOX cells are much more sensitive to staurosporine-induced apoptosis as compared to the drug-sensitive MCF-7 cells (see appendix for reprint by Devarajan et al., Int J Oncol, 2002).
5. The tTGase-expressing subclones in the stating tumor population may represent the future drug-resistant clones (see appendix for reprints by Devarajan et al. Int J Oncol, 2002 and by Mehta et al. J Natl Cancer Inst, 2002).
6. A significant majority of breast tumor samples from patients either lack or exhibit deficient caspase-3 expression (see appendix for preprint by Devarajan et al.).

Reportable outcomes:

Manuscripts:

1. Chen J, Aggarwal N, Mehta K. (2002) Multidrug-resistant MCF-7 breast cancer cells contain deficient intracellular calcium pools. Breast Cancer Res Treat 71: 237-247.
2. Devarajan E, Chen J, Multani A, Pathak S, Sahin A, Mehta K (2002) Human breast cancer MCF-7 cell line contains inherently drug-resistant subclones with distinct genotypic and phenotypic features. Int J Oncol 20: 913-20.
3. Mehta K, Devarajan E, Chen J, Sahin A, Multani A, Patak S (2002) Drug-resistant MCF-7 cells: an identity crisis? J Natl Cancer Inst, 94: 1653-1655.

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5. Chen, J, Multani A, Pathak S, Mehta K (2002) Drug-resistant MCF-7 cells are highly sensitive to apoptosis-inducing stimuli, (submitted).

Abstracts:

1. Chen SK, Mehta K (2002) Multidrug-resistant MCF-7 breast cancer cells are highly sensitive to calcium ionophore (A23187)-induced apoptosis. Proc Am Assoc Cancer Res 43: Abstr 4312.
2. Mehta K, Chen J, Devarajan E, Sahin A (2002) Significance of transglutaminase expression in drug-resistant breast cancer cells. 7th Int Conf Transglutaminase and Protein Crosslinking reactions, Ferrera, Italy (September 2002).

Presentations:

1. The University of Torino, Breast Cancer Program, Torino (Italy): Transglutaminase and multidrug resistance. (Host: Dr. Stefen Sapino) September, 2000.
2. Transglutaminase expression and drug-resistance: an arbiter or innocent bystander. The University of Rome, Italy: (Host, Prof. Mauro.Piacentini), June 8, 2001.

Cell Lines Developed:

1. Breast cancer MCF-7 cells transfected with active-site mutant transglutaminase.

Funding applied based on work supported by this award:

1. US Department of Defense – Idea Grant BC020178 (submitted, June 2002)

CONCLUSIONS

The development of drug-resistance in breast cancer cells is associated with increased expression of a multifunctional enzyme, tissue transglutaminase (tTGase). The breast cancer cells, in general, failed to sustain the expression of ectopic tTGase expression. Drug resistant cells have defective intracellular calcium pool that permits the survival of these cells in the phase of high tTGase expression. The clones with high levels of tTGase expression in the starting tumor population may represent inherently drug-resistant clones. tTGase may confer drug-resistance phenotype as a result of its ability to serve as a co-receptor for integrin-mediated binding to fibronectin and the activation of cell survival and anti-apoptotic signaling pathways.

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APPENDICES

Preprints:

1. Mehta K, Devarajan E, Chen J, Sahin A, Multani A, Pathak S (2002) Drug-resistant MCF-7 cells: an identity crisis? J Natl Cancer Inst, letter (In press).

2. Chen J, Aggarwal N, Mehta K. (2002) Multidrug-resistant MCF-7 breast cancer cells contain deficient intracellular calcium pools. Breast Cancer Res Treat 71: 237-247.

3. Devarajan E, Chen J, Multani A, Pathak S, Sahin A, Mehta K (2002) Human breast cancer MCF-7 cell line contains inherently drug-resistant subclones with distinct genotypic and phenotypic features. Int J Oncol 20: 913-20.

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APPENDIX 1

Multidrug-Resistant MCF-7 Cells: An Identity Crisis?

Two recent correspondences published in the Journal *(1,2)* labeled the MCF-7/ADR cell line—a multidrug-resistant (MDR) human breast cancer MCF-7 subline—as having a non-MCF-7 origin, which led to a change in the nomenclature of this cell line to NCI/ADR. We believe the original nomenclature of MCF-7/ADR should be retained.

Although the two MDR MCF-7 sublines (MCF-7/ADR and MCF-7 TH) used by the investigators whose work prompted the nomenclature change were independently established, they showed several genotypic and phenotypic similarities. Both contained a full-length functional caspase-3 protein *(2)*, despite complete loss of this protein in the parental MCF-7 cells because of a 47-base-pair deletion in exon 3 of the CASP-3 gene *(3)*. Interestingly, several features of the MCF-7/DOX subline established in our laboratory several years ago *(4)* were identical to those of the MCF-7/ADR and MCF-7 TH cells but different from those of the parental MCF-7 cells *(5)*. For example, similar to MCF-7/ADR and MCF-7 TH cells, the MCF-7/DOX cells showed high expression levels of P-glycoprotein (P-gp) and of a protein cross-linking enzyme, tissue transglutaminase; they also contained the full-length caspase-3 protein *(6)*. We thus sought to determine whether the development of drug resistance in MCF-7 cells represents selective selection and expansion of an inherently resistant

clone in the parental MCF-7 cell population. We purchased MCF-7 cells from the American Type Culture Collection (lot 2015862; Manassas, VA) and treated them with doxorubicin at 1 μg/mL. More than 99% of the MCF-7 cells died within 1 week, but a few colonies (an average of two colonies per T-75 flask were observed to grow in the presence of doxorubicin) of cells survived about 3 weeks of continuous culture in the presence of the drug. We expanded these colonies and, to our surprise, the newly established MCF-7 cell subline (MCF-7/WT/DOX) exhibited several biochemical features similar to those of the MCF-7/DOX and MCF-7/ADR cells but different from those of the parental MCF-7 cells. The MCF-7/WT/DOX cells showed high expression levels of both P-gp and tissue transglutaminase and contained full-length functional caspase-3 protein (Fig. 1, A). Karyotypic analysis of the MCF-7, MCF-7/DOX, and MCF-7/WT/DOX cell lines revealed unique features that were highly conserved in the drug-resistant sublines but were quite distinct in the parental MCF-7 cells *(5)*.

MCF-7 cells express a truncated isoform of caspase-3 transcript, whereas drug-resistant sublines express full-length caspase-3 transcript. Thus, we amplified the complementary DNA (cDNA) from MCF-7 and MCF-7/WT/DOX cells by polymerase chain reaction (PCR) using primers specific for full-length caspase-3 cDNA to further validate the presence of a drug-resistant clone in the parental MCF-7 cells. The resulting PCR products were analyzed for the presence of caspase-3 transcripts by agarose gel electrophoresis and ethidium bromide staining. The PCR products of the MCF-7 cells contained a predominant band corresponding to truncated caspase-3 transcript (Fig. 1, B); a minor band corresponding to full-length caspase-3 transcript became evident after many PCR cycles on the amplified cDNA (Fig. 1, B, arrow). The PCR-amplified products from the MCF-7/DOX cells showed only a single band, corresponding to the full-length caspase-3 transcript. These results confirmed the presence of one or more inherently resistant subclones in the parental MCF-7 cells that harbor the full-length CASP-3 gene and are likely to propagate into drug-resistant cell lines in the presence of MDR-related drugs. This possibility was further supported by our inability to establish any doxorubicin-resistant cell lines from two MCF-7 single-cell clones. These results appeared to be consistent with the findings that caspase-3-deficient MCF-7 cells, when reconstituted with caspase-3 cDNA, become more susceptible to chemotherapy-induced apoptosis *(6)*. It is likely that caspase-3-adequate MCF-7 subclones that propagate into drug-resistant sublines have additional pathways that confer selective resistance of these cells to chemotherapeutic agents. Despite their high resistance to doxorubicin, these cells are exquisitely sensitive to certain apoptosis-inducing agents. For example, Leoni et al. *(7)* observed that MCF-7/ADR cells were more sensitive to indanone-induced apoptosis than were the drug-sensitive MCF-7 cells. In our experience, the MCF-7/DOX cells were much more sensitive to a staurosporine-induced apoptosis than were the parental MCF-7 cells *(5)*.

Our research has demonstrated that drug-resistant MCF-7 cell lines result from parental MCF-7 cells that harbor the full-length CASP-3 gene. In view of these results, we suggest that the original nomenclature of MCF-7/ADR for MCF-7-derived drug-resistant sublines be retained to reveal the fact that various clones in a given tumor population can be extremely diverse in terms of their genotype and phenotypic characteristics.

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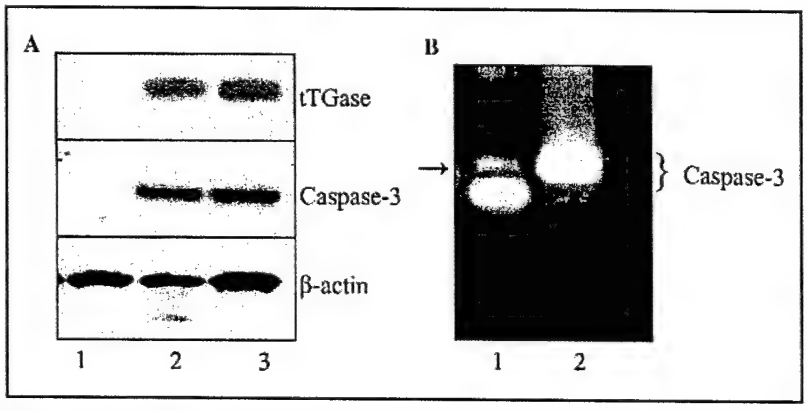

Fig. 1. Tissue transglutaminase (tTGase) and caspase-3 expression in MCF-7, MCF-7/WT/DOX, and MCF-7/DOX cell lines. **A)** tTGase and caspase-3 protein expression levels were examined by western blot analysis in the parental MCF-7 cells (purchased from American Type Culture Collection; **lane 1**), in drug-resistant MCF-7/WT/DOX cells (obtained by continuous culture of the parental MCF-7 cells in doxorubicin at 1 μg/mL; **lane 2**), and in MCF-7/DOX cells (obtained from Dr. Ken Cowan, National Cancer Institute; **lane 3**). **B)** Caspase-3 transcripts in MCF-7 cells (**lane 1**) and MCF-7/WT/DOX cells (**lane 2**), as determined after many polymerase chain reaction cycles of the complementary DNA from the respective cell lines. **Arrow** indicates the presence of full-length caspase-3 transcript in MCF-7 cells.

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NOTES

Editor's note: Dr. Scudiero declined to comment.

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RESPONSE

We read with great interest the correspondence by Mehta et al. These authors propose the hypothesis that the various doxorubicin (ADR)-selected MCF-7 sublines may result from the selective expansion of inherently resistant subclones that contaminate the parental MCF-7 cell line and that harbor the full-length CASP-3 gene. Although this theory is quite interesting, we have some caveats *(1)*. Because the CASP-3 gene is deleted in parental MCF-7 *(1)*, it is difficult to understand how this genotype could have been reverted in the hypothetically inherently resistant subclones *(2)*. In our original description, we assessed the nonidentity of MCF-7 and MCF-7 TH by DNA fingerprinting *(2)*. This method is more reliable than phenotypic karyotyping *(3)*. When they claim that an inherently resistant subclone contains functional caspase-3, Mehta et al. somehow contradict the finding that caspase-3 restores chemotherapy sensitivity in MCF-7 cells *(3)*.

Most importantly, Mehta et al. explain their hypothesis with a contamination of the parental MCF-7 cell line. Thus, we strongly caution against retaining the original nomenclature—MCF-7/ADR or MCF-7 TH—for these sublines of unknown origin. Good research cannot be performed with tools of uncertain identity.

FARZANEH PIRNIA
MARKUS M. BORNER

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Breast Cancer Research and Treatment **71:** 237–247, 2002.

APPENDIX 2

Report

Multidrug-resistant MCF-7 breast cancer cells contain deficient intracellular calcium pools

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Key words: apoptosis, calcium, multidrug resistance, thapsigargin, transglutaminase

Summary

Emergence of resistance to antineoplastic drugs poses a major impediment to the successful treatment of breast cancer. We previously reported that human breast carcinoma MCF-7 cells selected for resistance against doxorubicin (MCF-7/DOX cells) expressed high levels of tissue-type transglutaminase (tTGase), a calcium-dependent protein cross-linking enzyme that plays a role in apoptosis. The purpose of this study was to determine the mechanisms by which MCF-7/DOX cells survive and proliferate despite high levels of tTGase expression. Our results demonstrate that the MCF-7/DOX cells contain deficient intracellular calcium pools, which may explain their ability to survive and tolerate the high levels of tTGase expression. Treatment with thapsigargin failed to induce any significant killing of MCF-7/DOX cells. Similar treatment of the drug-sensitive MCF-7 wild-type (MCF-7/WT) cells, however, induced significant apoptosis. Treatment with the ionophore A23187, on the other hand, killed a large percentage of both the MCF-7/DOX and the MCF-7/WT cells. We also established a revertant cell line, MCF-7/RT, from MCF-7/DOX cells to rule out the involvement of P-glycoprotein (P-gp) in these phenomena. Unlike the MCF-7/DOX cells, the MCF-7/RT cells showed no detectable P-gp expression; the MCF-7/RT cells, however, continued to express high levels of tTGase. Moreover, like MCF-7/DOX cells, the MCF-7/RT cells were highly resistant to thapsigargin-induced apoptosis but were sensitive to the ionophore A23187-induced apoptosis. These results suggest that the resistance of MCF7/DOX cells to thapsigargin is linked to their defective intracellular Ca^{2+} stores, a notion that was directly confirmed by single-cell spectrofluorometric analysis.

Introduction

Doxorubicin is one of the most effective chemotherapeutic agents available for the treatment of breast cancer. The drug is able to induce regression of metastatic breast cancer when given alone [1, 2], and constitutes the core of most combination chemotherapeutic regimens currently used to treat breast cancer. The effectiveness of doxorubicin and several other anticancer drugs, however, is hindered by both intrinsic drug resistance and the development of drug-resistant tumor subpopulations.

A feature that is often associated with the development of drug resistance is expression of *mdr*, a gene that encodes for P-glycoprotein (P-gp), an integral transmembrane protein [3]. The main function of P-gp is to enhance drug efflux, resulting in a net decrease in the intracellular accumulation of drugs [4–6]. Although P-gp overexpression is the most-studied and best-characterized mechanism of resistance to many drugs, P-gp is unlikely to account completely for the drug resistance seen in many tumors. A decreased expression of type II topoisomerase has been proposed as the mechanism for non-P-gp mediated drug resistance in tumors in many models [7]. Similarly, altered expressions of other enzymes, such as type I topoisomerase [8], glutathione-related enzymes [9], and protein kinase C (PKC) [10] have been observed during the transition from drug sensitivity to drug resistance.

It has been reported that protein cross-linking enzyme tissue transglutaminase (tTGase) is overexpressed in MDR^+ breast cancer (MCF-7) [11] and lung cancer (PC-14) [12] cells selected for resistance

to doxorubicin. Tissue TGase (EC 2.3.2.13) is an 80-kDa enzyme that catalyzes Ca^{2+}-dependent cross-links between the γ-carboxamide group of a peptide-bound glutamine residue and the primary amine group of a variety of molecules [13, 14]. The resultant γ-(ε-glutamyl) lysine isopeptide bonds are highly stable and resistant to proteolytic and detergent degradation [15]. Several studies have implicated tTGase-catalyzed reactions in the process of apoptosis [16–21]. The induction and activation of tTGase in cells undergoing apoptosis lead to an irreversible cross-linking of proteins, thus preventing the leakage of intracellular macromolecules that are believed to play a role in averting inflammation and in autoimmunity [22, 23]. Furthermore, forced expression of tTGase in several cell types increased the rate of spontaneous apoptosis or rendered these cells highly susceptible to various death-inducing stimuli [24–26]. These observations suggest that cells do not readily tolerate tTGase and that its induction and activation may be critical events in apoptosis.

Our previous work demonstrated a significant increase in the level of tTGase expression in doxorubicin-resistant MCF-7 (MCF-7/DOX) cells [11]. These cells exhibit the multidrug resistance (MDR) phenotype and are greater than 150 times more resistant to doxorubicin-induced cell-death than are wild-type MCF-7 (MCF-7/WT) cells. The purpose of the current study is to determine the mechanism(s) that enable MCF-7/DOX cells to survive and proliferate despite high levels of tTGase expression. Our results suggest that MCF-7/DOX cells carry defective intracellular calcium pools that protect the cells from the toxic effects of tTGase. Moreover, because doxorubicin-induced alteration in cytosolic free-calcium ($[Ca^{2+}]i$) is critical for drug-mediated cell death [27], deficient intracellular calcium pools in MCF-7/DOX cells may represent a novel mechanism of drug resistance.

Materials and methods

Cell lines

MCF-7/DOX cells were derived from the parental, drug-sensitive MCF-7 cells by stepwise selection with doxorubicin [11]. The other cell lines – U937/DOX human monoblastic leukemia (MDR^+; selected for resistance against doxorubicin) and U937/VCR (MDR^+; selected for resistance against vincristine) – were kindly provided by Dr Panayotis Pantazis (The Stehlin Foundation for Cancer Research, Houston, TX) [28]. The drug-sensitive (CT26) and doxorubicin-resistant (CT26/DOX), murine colon carcinoma cell lines and ultraviolet (UV)-induced fibrosarcoma (UV2237), and the drug-sensitive (UV2237) and doxorubicin-resistant (UV2237/DOX) cell lines were kindly provided by Dr Isaiah Fidler (The University of Texas M.D. Anderson Cancer Center).

To maintain the drug-resistance phenotype, all cell lines were cultured in the presence of 1 μg/ml doxorubicin. A revertant MCF-7 cell subline (MCF-7/RT) was established by culturing MCF-7/DOX cells in the absence of doxorubicin. After about 6 months, the P-gp expression became undetectable in MCF-7/RT and the cells had regained partial sensitivity to doxorubicin-induced killing. All the cell lines were maintained at 37°C in 5% CO_2/95% air in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal calf serum (Sigma Chemical Co., St. Louis, MO), 10 mM glutamine, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin).

Cell viability

The number of viable cells remaining after treatment with various agents was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co.) reduction assay [29]. Briefly, cells were plated (4,000 cells/well per 0.2 ml RPMI 1640 medium) in 96-well microtiter plates and incubated overnight. The test agent was then added at indicated concentrations to triplicate wells. After 48 h, MTT was added to each well at a final volume of 0.5 mg/ml, and microplates were incubated at 37°C for 3 h. After the supernatant was removed, the formazan salt resulting from the reduction of MTT was solubilized in dimethyl sulfoxide (DMSO; Sigma Chemical Co.) and the absorbance was read at 570 nm using an automatic plate reader (Molecular Devices Corporation, Sunnyvale, CA). The cell viability was extrapolated from optical density $(OD)_{570}$ values and expressed as percent survival using the following formula:

$$\%\ \text{cell viability} = \frac{OD_{570}\ \text{of drug treated sample}}{OD_{570}\ \text{of untreated control sample}} \times 100$$

tTGase activity

Cell monolayers at 70–80% confluency were washed thoroughly in phosphate-buffered saline (PBS; 0.1 M,

pH 7.4), collected, and resuspended in a minimal volume (100–300 μl) of lysis buffer (20 mM Tris–HCl, pH 7.4, containing 1 mM EDTA, 150 mM NaCl, 14 mM 2-mercaptoethanol, and 1 mM phenylmethylsulphonyl fluoride [PMSF]). Cells were then lysed in the same buffer by probe sonication for 8–10 pulses of 10 s each. In some instances, nuclei were separated from the cytosolic fraction by incubating cells on ice for 20 min in a hypotonic HFPES-KOH buffer (10 mM, pH 7.4). The cells were broken with a glass Dounce homogenizer, and the nuclei were isolated as described previously [30]. The protein content of the cell lysates was determined using the dye reagent (BioRad, Richmond, CA). Cell lysates were then assayed for tTGase activity by determining the Ca^{2+}-dependent incorporation of [^{3}H]-putrescine (specific activity 14.3 Ci/mmol, Amersham-Pharmacia, San Francisco, CA) into dimethyl casein [11]. The enzyme activity was expressed as nanomoles of putrescine incorporated per hour per milligram total lysate protein.

Western blot analysis

To solubilize the cell proteins, the cell lysates were mixed with 3× sample buffer (60 mM Tris–HCl [pH 6.8], 10% [vol/vol] glycerol, 2% sodium dodecyl sulfate [SDS], 5% [vol/vol] β-mercaptoethanol, and 0.0025% bromophenol blue) and boiled for 3 min. Thirty micrograms of protein were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGF) on a 7.5% gel and electrophoretically transferred to a nitrocellulose membrane (Amersham-Pharmacia) using a semidry transfer apparatus (Hoefer Scientific Instruments, San Francisco, CA). The membrane was blocked overnight in 5% (wt/vol) nonfat dry milk in PBS (pH 7.4) containing 0.1% Tween-20 (PBS-T). The membranes were incubated with either tTGase monoclonal antibody (CUB7401; Neomarkers, Fremont, CA) at 1:3,000 dilution or with P-gp antibody (C219; DAKO Corporation, Carpinteria, CA) at 1:200 dilution. The Amersham-Pharmacia ECL system was used to detect the reaction between antigen and antibody. Western blot analysis was also employed to quantitate tTGase protein levels in MCF-7 cell extracts. Briefly, internal standards containing 0.05–1.0 μg of purified guinea pig liver TGase (Sigma Chemical Company) were run in parallel lanes. To calculate the amount of tTGase in cell extracts, the density of the immunoreactive band was determined by densitometry and interpolated on the line of standards.

Northern blot analysis

To determine tTGase expression at the messenger-RNA level, total RNA was isolated from cells during the logrithmic-phase of cell growth using TRIZOL reagent (Invitrogen Corparation, Carlsbad, CA), according to the manufacturer's instructions. Samples containing 15 μg total RNA were separated on 1% formaldehyde agarose gel and transferred onto a Nytran membrane (Schleicher and Schuell, Hayward, CA). The membrane was prehybridized with ^{32}P-labeled tTGase cDNA probe generated by nick translation of tTGase cDNA clone pTG3400, which was kindly provided by Dr Peter Davies (The University of Texas Medical School, Houston TX). Blots were washed in 2× SSC and autoradiographed on Kodak XAR-5 film (Eastman Kodak Company, Rochester, NY) at −70°C.

Confocal microscopy

To examine the intracellular distribution of tTGase protein in MCF-7/DOX cells, we used scanning confocal laser microscopy (Molecular Dynamics, Sunnyvale, CA). After 2 days of culture on coverslips, the cells were washed with PBS, fixed in 4% formaldehyde, and permeabilized by treatment with 0.3% Triton X-100 for 10 min at room temperature. The coverslips were rinsed in PBS, blocked for 30 min in blocking buffer (PBS containing 25% normal rabbit serum and 5% bovine serum albumin) and then incubated for 1 h with CUB7401 monoclonal antibody (0.1 μg/ml). The coverslips were washed three times with PBS-T and incubated with fluorescein isothiocynate-conjugated anti-mouse immunoglobulin G (IgG) secondary antibody for 45 mm. The cover slips were washed in serum-free medium and inverted on glass slides onto a drop of 50% glycerol-PBS containing 0.2% p-phenylenediamine. The cells were then visualized using a Molecular Dynamics confocal microscope system and argon laser scanner.

Measurement of cytosolic free calcium ([Ca^{2+}]i)

[Ca^{2+}]i was measured using the INCa workstation system (Intracellular Imaging, Inc., Cincinnati, OH) by recording the Fura-2 fluorescence at excitation and emission wavelengths of 340 and 50 nm, respectively. Briefly, 5×10^4 cells were seeded onto sterilized microscopic coverslips 2 days before the experiment. The coverslips containing 70–80% confluent monolayers of cells were removed, washed, and incubated at 37°C for 1 h in a serum-free medium containing

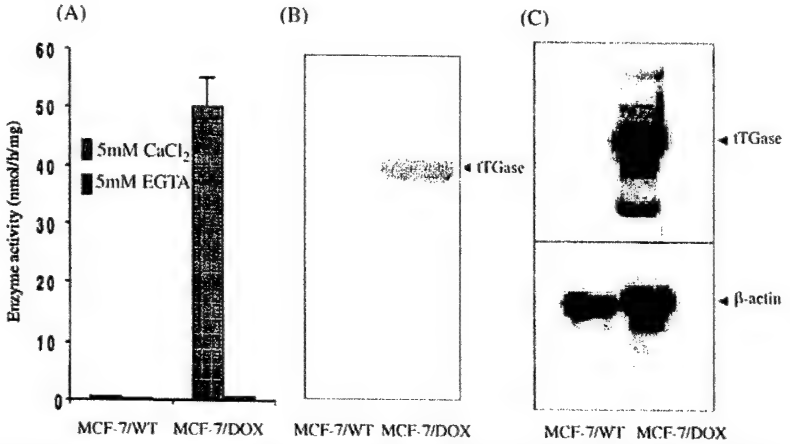

Figure 1. tTGase expression in drug-sensitive (WT) and drug-resistant (DOX) human breast carcinoma (MCF-7) cell lines. (A) The enzymatic activity was determined in cell lysates by studying calcium-dependent incorporation of [^{3}H]-putrescine into dimethyl casein as described in Materials and Methods. The enzyme-specific activity was calculated from trichloroacetic acid precipitable counts incorporated into dimethyl casein in the presence of calcium. Results shown are the means ± standard deviation of at least six values. (B) Western blot analysis for tTGase was carried out by fractionating 30 μg of cell lysates on SDS-PAGE and probing the nitrocellulose membranes with anti-tTGase antibody. A peroxidase-conjugated anti-mouse immunoglobulin G (IgG) detection system was used to detect the immunoreactivity. (C) Northern blot analysis to determine tTGase mRNA expression in drug-sensitive (WT) and drug-resistant (DOX) MCF-7 cells was carried out as described in Materials and Methods. The membrane was then reprobed with β-actin selective probe to ascertain even loading.

2 μM Fura-2/AM (Sigina Chemical Company) and 0.02% (wt/vol) pluronic acid (F-127; Sigma Chemical Company). After Fura-2 was removed and the coverslips were washed in calcium-free Krebs–Ringer bicarbonate medium, the cells were monitored under UV light. The intensity of the emitted fluorescence was monitored at 340 and 380 nm using two photomultiplier tubes, the output of which was sampled by computer. The ratio of the fluorescence intensity at 340 nm to the fluorescence intensity at 380 nm was then calculated. These ratio values were used to interpolate the calcium concentrations according to an *in vitro* calibration curve. To determine the levels of inducible calcium in the $[Ca^{2+}]i$ pools, ionomycin or thapsigargin (both from Sigma) were added, each at a final concentration of 1 mM. The levels of $[Ca^{2+}]i$ released in response to either reagent treatment were determined as described above. Finally, $CaCl_2$ was added at a final concentration of 1 mM to ascertain the loading of cells with Fura-2. The data were then analyzed using the INCA software.

Results

Expression of tTGase in drug-resistant cells

The MCF-7/DOX cells exhibited strong tTGase activity (48 ± 5 nmol/h/mg) as previously observed [11]. In contrast, the drug-sensitive MCF-7 cells had very weak or undetectable tTGase activity (2 ± 0.5 mmol/h/mg), as demonstrated by their poor ability to incorporate [^{3}H]-putrescine into dimethyl casein (Figure 1(A)). The high enzyme activity in the MCF-7/DOX cells was associated with an increased expression of the tTGase protein as detected using western blot analysis (Figure 1(B)). A semiquantitative method using western blot analysis revealed that the tTGase protein in the MCF-7/DOX cells constitutes as much as 4% of the total cell protein. No detectable tTGase protein was observed in the drug-sensitive MCF-7 cells. The large increase in tTGase activity and protein accumulation in the MCF-7/DOX cells also corresponded to the large accumulation of tTGase mRNA in these cells, as revealed by northern blot analysis (Figure 1(C)).

To evaluate the intracellular distribution of tTGase in the MCF-7/DOX cells, we performed confocal microscopy of the immunostained cells. The MCF-7/DOX cells were strongly immunoreactive to anti-tTGase antibody (Figure 2). This strong reactivity was predominantly localized in the cytosolic compartment, and a small amount of immunoreactivity was detected in the nuclei. Next, we separated the cytosolic and nuclear fractions from MCF-7/DOX cells to analyze the distribution of the enzyme activity in the two fractions. Five percent or less of the total activity was

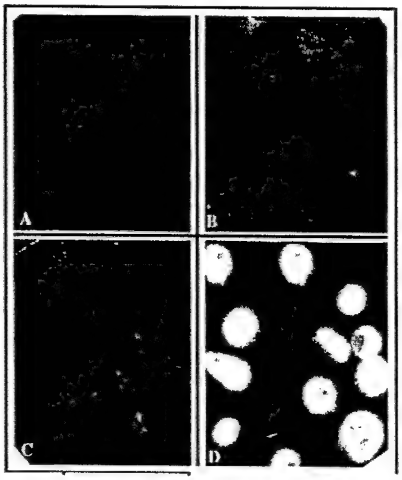

Figure 2. Confocal microscopic images of MCF-7/WT (A and B) and MCF-7/DOX (C and D) cells immunostained with the control IgG_1 (A and C) or tTGase-specific anti-IgG_1 (B and D).

associated with the nuclear fraction, whereas, 95% or more of the activity was associated with the soluble fraction (data not shown).

We next sought to determine whether the elevated level of tTGase is selective for MCF-7/DOX cells, or is a general phenomenon associated with the acquisition of the drug-resistance phenotype. Results shown in Table 1 suggested that regardless of their origin, the cancer cells when selected for resistance against MDR-related drugs exhibited increased tTGase activity. The increased enzyme activity in drug-resistant cells was associated with a parallel increase in tTGase protein levels, as revealed by western blot analysis (data not shown). Interestingly, the level of tTGase activity and expression correlated with the level of resistance to doxorubicin. These observations suggest that tTGase may be an important feature of the MDR phenotype of tumor cells.

Effect of cytosolic Ca^{2+} on apoptosis of MCF-7 cells

The protein cross-linking activity of tTGase depends completely on the presence of Ca^{2+} [31]. In view of previous observations that tTGase expression is associated with apoptotic events in various cell types [16–21], it was reasonable to speculate that MCF-7/DOX cells might contain deficient or defective intracellular pools of calcium. To test this contention, we first determined the effect of a pharmacologic agent, thapsigargin, on the survival of MCF-7/WT and MCF-7/DOX cells. By selectively inhibiting the calcium-pumping ATPase in the endoplasmic reticulum, thapsigargin depletes the intracellular Ca^{2+} stores, thereby increasing cytosolic $[Ca^{2+}]i$. Treatment of the MCF-7/WT cells with thapsigargin caused substantial cell death in a dose-dependent manner (Figure 3(A)). Interestingly, treatment of the MCF-7/DOX cells under similar conditions failed to induce any apparent damage, as revealed by the cell' ability to reduce MTT into an insoluble formazan (Figure 3(A)). In contrast, treatment with the ionophore A23187, which permits Ca^{2+} influx from an extracellular pool, resulted in a significant cytotoxicity in both the MCF-7/WT and the MCF-7/DOX cells (Figure 3(B)). These observations suggested that the drug-sensitive and drug-resistant MCF-7 cells are equally sensitive to the cytotoxic effects of increased concentration of $[Ca^{2+}]i$. Interestingly, the cell death induced by $[Ca^{2+}]i$ was apoptotic, as revealed by internucleosomal 'DNA laddering' patterns obtained from the A23187- or thapsigargin-treated MCF-7/WT cells and the A23187-treated MCF-7/DOX cells (Figure 3(C)). The resistance of MCF-7/DOX cells to thapsigargin may be related to the defective release of Ca^{2+} from the cells' intracellular pools or, alternatively by, mediation of the efflux of thapsigargin in these cells by P-gp. Indeed, previous studies have suggested that thapsigargin can serve as a substrate for P-gp and can be effectively pumped out by MDR^+ cells [32].

Resistance of MCF-7/DOX cells to thapsigargin in absence of P-gp

To further define the involvement of P-gp in the thapsigargin resistance of MCF-7/DOX cells, we established an MCF-7/DOX subline. After 6 months of continuous culture of MCF-7/DOX cells in doxorubicin-free medium, the MCF-7/DOX cells' sensitivity to doxorubicin was partially restored (Figure 4(A)). Interestingly, the P-gp expression in these MCF-7/RT cells became undetectable by western blot analysis (Figure 4(B)). However, the tTGase expression levels remained similar to those in the parental MCF-7/DOX cells (Figure 4(C)) We then used the MCF-7/RT cells to determine whether the thapsigargin resistance displayed by the MCF-7/DOX cells (Figure 3(A)) was due to P-gp-mediated efflux of thapsigargin. Despite a complete lack of p-gp expression, the MCF-7/RT cells exhibited substantial resistance to thapsigargin (Figure 5(A)). Moreover, cyclosporin A (CsA) and verapamil (Vp), the two well-known inhibitors of

Table 1. Transglutaminase activity in drug-sensitive and drug-resistant cell lines

Cell line	Enzyme activity (nmols/h/mg)	Relative resistance[a]	P-gp expression
Human			
MCF-7 (breast carcinoma)	2.2 ± 0.3	1.0	–
MCF-7/DOX[b]	42.3 ± 3.2	180.0	+++
U937 (monoblastic)	1.7 ± 0.2	1.0	–
U937/DOX200[b]	12.6 ± 1.4	200.0	++
U937/DOX400[b]	31.0 ± 4.0	400.0	++
U937/VCR40[c]	6.7 ± 0.5	98.0	+
U937/VC200[c]	10.2 ± 1.03	250.0	++
Murine			
CT26 (colon carcinoma)	1.8 ± 0.3	1.0	–
CT26/DOX[b]	6.0 ± 0.5	330.0	+++
UV2237 (fibrosarcoma)	3.9 ± 0.4	1.0	–
UV2237/DOX[b]	14.5 ± 0.3	100.0	+++

[a]Relative resistance is expressed as the IC_{50} of the resistant cell line against doxorubicin divided by the IC_{50} of the parental drug-sensitive cell line.
[b]Selected against doxorubicin.
[c]Selected against vincristine.
The expression of P-gp was determined by western blot analysis. Immunoreactive band intensity: +++, high; ++, intermediate; +, low; −, undetectable.

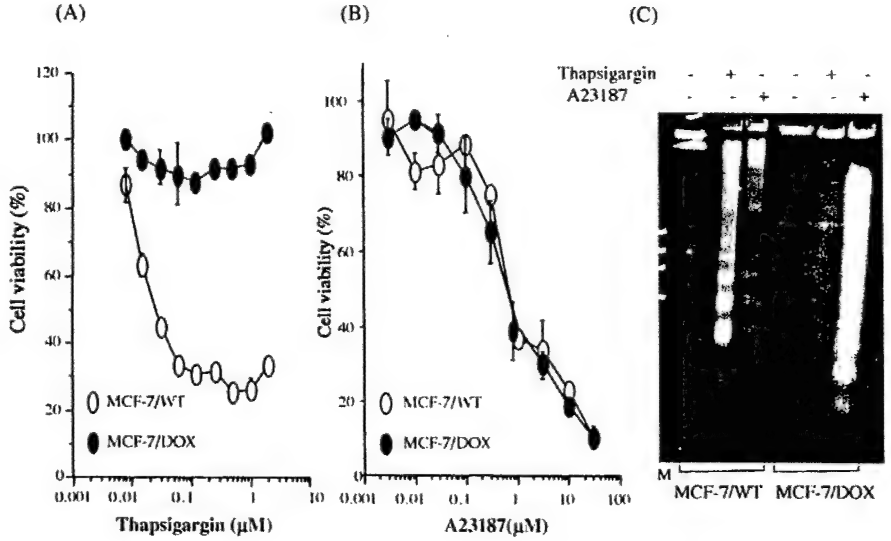

Figure 3. Thapsigargin and ionophore A23187-induced cytotoxicity in MCF-7/WT and MCF-7/DOX cells. Cells were seeded in 96-well plates 24 h prior to treatment with increasing doses of thapsigargin (A) or ionophore A23187 (B). After 48 h of treatment, cell survival was determined using MTT reduction assay as described in Materials and Methods. Values represent the mean percent survival ± standard deviation from at least three independent experiments compared with untreated control cells (100% survival). (C) Thapsigargin- and A23187-induced death of MCF-7/WT and MCF7/DOX cells is associated with internucleosomal degradation of the DNA. Cells were incubated in medium alone or medium containing 1 μM thapsigargin or 1 μMA23187. Forty-eight hour later, the cells were harvested, and low-molecular-weight DNA from 2×10^6 cells was isolated and analyzed on agarose gel. M indicates a 1 kb DNA ladder standard.

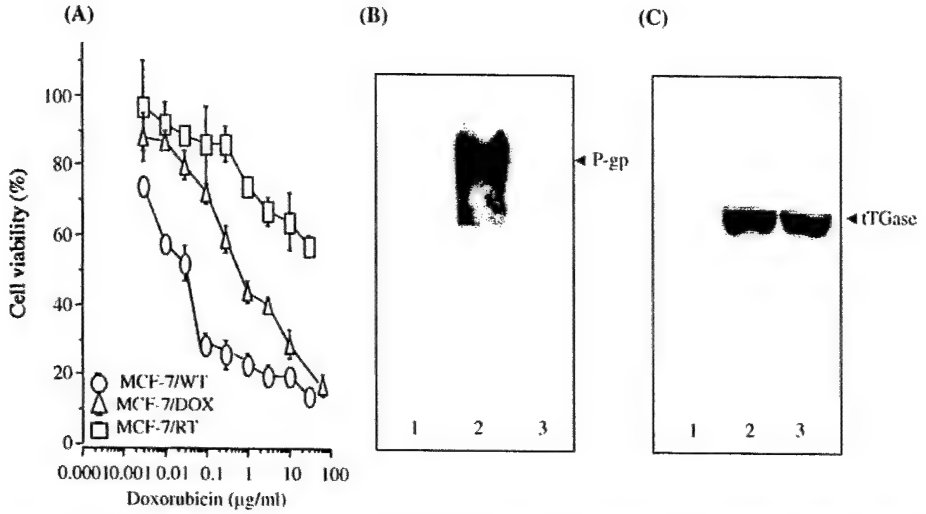

Figure 4. Characterization of the MCF-7/RT cell line. The MCF-7/RT cell subline was established by continuous culture of MCF-7/DOX cells in the absence of doxorubicin, as detailed in Materials and Methods. After 6 months of culture in the drug-free medium, the cells were tested for their sensitivity to doxorubicin (A) as well as for P-gp (B) and tTGase (C) protein expression and compared with the parental MCF-7/DOX and MCF-7/WT cells. The MCF-7/RT cells lacked detectable P-gp expression (B) but continued to express tTGase (C). A shows the cell survival values (mean ± standard deviation) from two independent experiments, each performed in triplicate. In B and C, lane 1 represents lysate from MCF-7/WT cells; lane 2, lysate from MCF-7/DOX cells; and lane 3, lysate from MCF-7/RT cells.

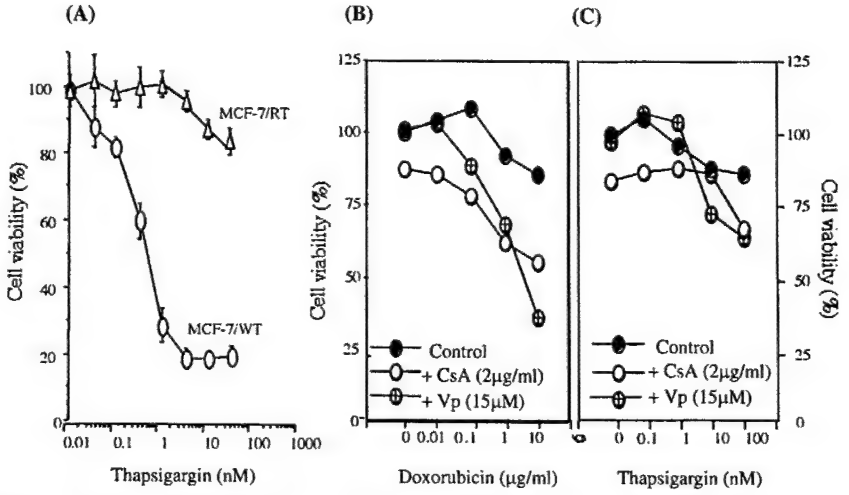

Figure 5. Resistance to thapsigargin-induced cell death is independent of P-gp expression. MCF-7/RT cells were treated with increasing doses of thapsigargin (A) under conditions similar to those described in the Figure 3 legend. At the end of treatment (48 h), cell viability was determined by MTT assay. Similarly, MCF-7/DOX cells were incubated in the presence of increasing concentrations of either thapsigargin alone (B) or doxorubicin alone (C) or in combination with P-gp inhibitors, cyclosporin A (2 μg/ml) or verapamil (15 μM). After 48 h of incubation under appropriate conditions, the cells were analyzed for viability using MTT assay. The values shown (mean ± standard deviation) are from a representative experiment performed at least three times with similar results.

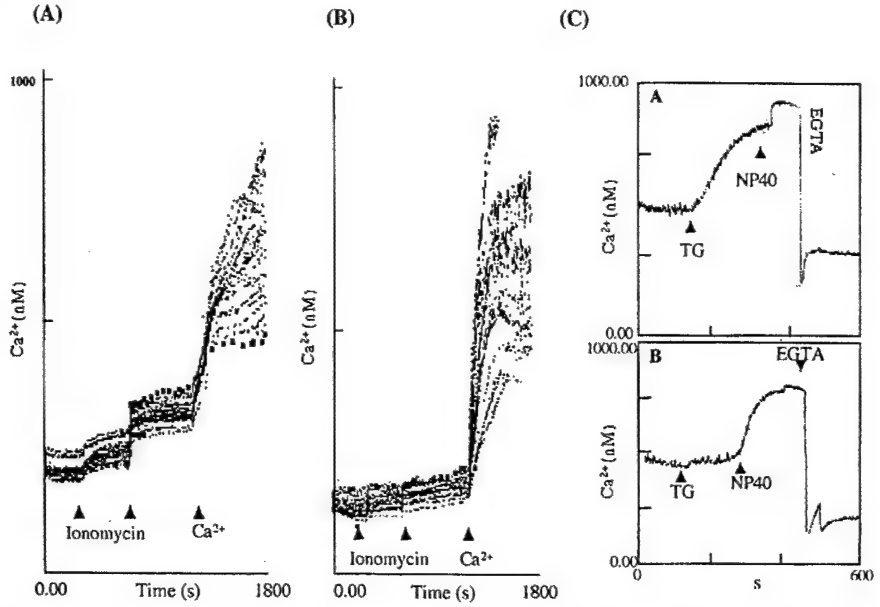

Figure 6. Spectrophotometric analysis of ionomycin- and thapsigargin-induced calcium fluxes in MCF-7 cells. MCF-7/WT (A) or MCF7/DOX cells (B) were labeled with Fura-2, as described in Materials and Methods, and fluorescence was monitored over time in response to ionomycin (A and B) or thapsigargin (C) treatment in a Ca^{2+}-free buffer. Ionomycin, $CaCl_2$, NP4O (0.4% final concentration), or EGTA (50 mM final concentration) were added at the indicated time points.

P-gp, failed to reverse thapsigargin resistance (Figure 5(C)), although both drugs partly reversed the doxorubicin resistance under similar conditions (Figure 5(B)), suggesting that under the experimental conditions employed both agents are able to inhibit P-gp functions. These observations led us to conclude that P-gp may not be entirely responsible for the observed resistance of MCF-7/DOX cells against thapsigargin and supported the contention that defective or deficient intracellular Ca^{2+} pools may contribute to this phenomenon.

Defective or deficient intracellular Ca^{2+} pools in MCF-7/DOX cells

We used Fura-2 spectrofluorometric analysis to determine the effect of either ionomycin-induced or thapsigargin-induced changes in $[Ca^{2+}]i$ in MCF-7/DOX cells. In this experiment, ionomycin and thapsigargin were selected to induce calcium release because, unlike A23187, these agents are not self-fluorescent. In the absence of external Ca^{2+}, the addition of ionomycin to MCF-7/WT cells induced a time-dependent increase in $[Ca^{2+}]i$ from approximately 200 to 350 nM over a course of 20 min, as determined from the fluorescence intensity (Figure 6(A)). The identical experimental conditions in the MCF-7/DOX cells, however, induced only a marginal increase in $[Ca^{2+}]i$. For example, the increase in $[Ca^{2+}]i$ in the ionomycin-treated MCF-7/DOX cells was only from 160 to 200 nM (Figure 6(B)). The addition of $CaCl_2$ to the bathing buffer immediately increased the $[Ca^{2+}]i$ in both cell types to comparable levels, suggesting an even loading of Fura-2 dye (Figure 6). The results shown in Figure 6(C) demonstrate that MCF-7/DOX cells exhibit a similar defect in thapsigargin-induced intracellular Ca^{2+} pool.

Discussion

The data presented here suggest that MCF-7/DOX cells exhibit deficient intracellular Ca^{2+} pools. These deficient pools may render tTGase inactive, prevent apoptosis, and enhance the survival of MCF-7/DOX cells despite overexpression of the tTGase enzyme. The transglutaminase enzyme family, of which tTGase is a member, catalyzes the calcium-dependent post-translational modification of proteins by establishing highly stable isopeptide bonds that make newly formed polymers resistant against mechanical and chemical insults. In fact, the only way

TGase-catalyzed protein polymers can be destroyed is by proteolytic digestion of the protein chains [15]. Although the exact physiologic function of tTGase remains unknown, its catalytic action has been implicated in several biologic processes, including cross-linking of integral plasma membrane proteins with the cytoskeleton. Studies have shown that tTGase also facilitates apoptosis [14, 16–19]. In most cell types, tTGase expression levels are very low but are markedly increased during apoptosis. Moreover, the forced expression of tTGase by transfection induces spontaneous apoptosis or renders the cells highly sensitive to apoptotic stimuli in several cell types [21, 24, 33]. Conversely, the reduction of tTGase levels by the use of antisense renders the cells resistant to apoptosis [24]. The tTGase enzyme appears to be activated by an elevation in the concentration of $[Ca^{2+}]i$, which is essential for apoptosis in many cell types [34].

Our previous studies demonstrated that MCF-7/DOX exhibit a dramatic increase in tTGase expression levels [11]. This observation was recently confirmed and extended by Han and Park [12], who observed that the tTGase expression level in doxorubicin-resistant lung cancer (PC-14/ADR) cells was 10 times higher than that in the drug-sensitive PC-14 cells. The reduction in tTGase levels by stable transfection with the tTGase-specific antisense or ribozyme made the PC-14/ADR cells sensitive to not only MDR-related drugs (doxorubicin or vincristine) but also non-MDR-related drugs (cisplatin). Thus, these authors concluded that tTGase may play a role in the acquisition of drug resistance.

After elaborating on these observations and studying several MDR^+ and MDR^- cell lines, we conclude that cells selected for the drug resistance phenotype consistently exhibit increased tTGase expression levels (Table 1). Although other investigators previously observed that tTGase overexpression can induce apoptosis, we sought to determine the mechanisms that could contribute to the successful survival of MDR^+ cells in the presence of increased tTGase expression levels. We used MCF-7/DOX cells as a model to address this issue because these cells express extremely high levels of the tTGase enzyme (approximately 4% of the total cell protein (Figures 1 and 2). Because the protein cross-linking function of tTGase is completely dependent on the presence of calcium, we first tested the effect of a pharmacologic agent, thapsigargin, on the survival of drug-sensitive and drug-resistant MCF-7 cells. Thapsigargin treatment increases cytosolic Ca^{2+} by inhibiting the calcium-pumping ATPase in endoplasmic reticulum membranes and thus emptying the intracellular pools of Ca^{2+} [35]. When treated with thapsigargin the MCF-7/WT cells underwent substantial apoptosis, but the MCF-7/DOX cells exhibited complete resistance to thapsigargin-induced cell death (Figures 3(A) and (C)). Because the P-gp inhibitors CsA and Vp failed to reverse thapsigargin resistance in the MCF-7/DOX cells (Figure 5(B)) and because the MCF-7/RT cells, which lacked P-gp expression (Figure 4(B)), continued exhibiting resistance to thapsigargin (Figure 5(A)), we concluded that the resistance of MCF-7/DOX cells to thapsigargin was not a P-gp-mediated event. Interestingly, an increase in cytosolic Ca^{2+} levels from an extracellular pool as a consequence of treatment with ionophore A23187 rendered all three MCF-7/WT, MCF-7/DOX, and MCF-7/RT cell lines equally sensitive to apoptosis (Figures 3(B) and (C)). These results suggest that MCF-7/DOX and MCF-7/RT cells may contain defective or deficient intracellular Ca^{2+} pools. We confirmed this contention by demonstrating that the ionomycin- and thapsigargin-sensitive calcium pools are defective in MCF-7/DOX cells (Figure 6). These observations suggest that the enzyme tTGase is present in drug-resistant cells as an inactive enzyme and thus may exhibit resistance to its apoptosis-inducing effect. Indeed, calcium is essential for the activation of tTGase and for the formation of a detergent-insoluble cross-linked protein scaffold in cells undergoing apoptosis [34, 36].

Deficient intracellular Ca^{2+} pools in MDR^+ cells may represent an important mechanism of the cells' ability to sustain cytotoxic effects of certain chemotherapeutic agents. It is well known that under normal conditions, the $[Ca^{2+}]i$ in resting cells is maintained at a nanomolar level. The calcium concentration is much higher (millimolar level), however, in the extracellular milieu and inside the endoplasmic reticulum. The treatment of cells with cytotoxic agents such as doxorubicin can generate reactive oxygen species and thus damage the calcium transport system localized in the endoplasmic reticulum, mitochondria, and plasma membranes. This damage leads to a disruption of calcium homeostasis, a sustained increase in $[Ca^{2+}]i$ and the onset of apoptosis [36, 37]. Thus, deficient intracellular Ca^{2+} pools may permit MCF-7/DOX cells to sustain free radical-induced damage, representing a novel mechanism of drug resistance in these cells. Indeed, it has been suggested that hydrogen peroxide too is a key mediator for doxorubicin-induced DNA fragmentation and apoptosis [38]. These obser-

vations suggest that, at least in MCF-7/DOX cells, a deficiency of intracellular Ca^{2+} pools may make cells resistant to tTGase-induced apoptotic events and, more importantly, may render these cells sensitive to certain apoptotic agents.

We do not know if increased levels of tTGase expression play any role in the acquisition of drug resistance by the tumor cells. Recent work by Han and Park [12] suggests that tTGase may contribute to drug resistance. These authors concluded that tTGase can facilitate drug resistance through some general mechanism rather than through the P-gp-related system. Moreover, in addition to catalyzing the Ca^{2+}-dependent cross-linking reactions, tTGase can also function as a signal-transducing G-protein [39–41]. Thus, the 74-kDa α-subunit of the G-protein (Gα) associated with a 50-kDa β-subunit of the GTP-binding protein G_h is a tTGase [39–42]. The dimer acts in association with the α_1-adrenergic receptor in a ternary complex and activates phospholipase C (PLC) [43, 44]. The activated PLC in turn hydrolyzes phosphatidylinositol diphosphate to generate the second messengers inositol triphosphate and diacylglycerol. Inositol triphosphate induces Ca^{2+} release from intracellular pools [45]. Diacylglycerol activates PKC [46], a serine threonine kinase that influences many biologic processes, including regulation of the multidrug resistance phenotype [47]. In general, the MDR^+ cells express higher levels of PKC activity, which, by phosphorylating the P-gp, renders these cells resistant to some chemotherapeutic agents. It is not known whether tTGase can affect drug resistance by activating PLC or some other pathway. However, our current laboratory studies on transfection of MCF-7/WT cells with the full-length tTGase cDNA and a mutant form of cDNA, wherein a serine substitutes for the active site cysteine (Cys277), should address whether protein cross-linking or signaling or both tTGase activities may contribute to the acquisition of drug resistance by the tumor cells.

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 APPENDIX 3

Human breast cancer MCF-7 cell line contains inherently drug-resistant subclones with distinct genotypic and phenotypic features

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Abstract. The resistance of cancer cells to multiple chemotherapeutic agents poses a major problem in the successful treatment of breast cancer. Whether drug resistance is due to changes induced in the drug-exposed tumor cells or represents the selective growth of one or more drug-resistant clones present in the initial tumor remains controversial. Here we provide evidence that the development of multidrug resistance in a human breast cancer cell line (MCF-7) is a result of propagation of an inherently resistant subclone. The drug-resistant MCF-7 (MCF-7/DOX) cells exhibited several phenotypic and genotypic features that were notably distinct from those observed in the parental drug-sensitive (MCF-7/WT) cells. The most striking change was the presence of a full-length functional caspase-3 in MCF-7/DOX cells that was missing in the parental MCF-7/WT cells due to a deletion mutation in the caspase-3 gene. A drug-resistant MCF-7 cell subline (MCF-7/WT/DOX) was established by exposing the MCF-7/WT cells directly to a high dose of doxorubicin and used for determining the phenotypic and genotypic alterations associated with drug resistance. The MCF-7/WT/DOX cells exhibited alterations identical to those of the MCF-7/DOX cells but which were strikingly distinct from the parental MCF-7/WT cell line. These results suggest that drug resistance is an inherent property of some cancer cells that are present in the initial tumor burden and exhibit distinct phenotypic/genotypic alterations.

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Abbreviations: MCF-7/DOX, multidrug-resistant MCF-7 cells; MCF-7/WT, drug-sensitive MCF-7 cells; MDR, multidrug resistance; P-gp, P-glycoprotein; SSP, staurosporine; tTGase, tissue-type transglutaminase; UM, unidentified marker chromosomes

Key words: breast cancer, caspase-3, apoptosis, drug resistance, transglutaminase

Introduction

Breast cancer is the most common malignancy among women in the Western world (1). Most patients, who attain chemotherapy-induced remission, relapse and eventually die as a result of the emergence of subpopulations of tumor cells that are resistant to drugs (2). The resistance is not confined to drugs used to treat the tumor initially; it extends to agents to which the patient has not been exposed and that share no obvious structural similarities with the drug used in the initial treatment. Little information is available about the causes of this cross-drug resistance in breast cancer. It is not clear whether the drug resistance results from induced changes in the drug-exposed tumor cells (adaptation) or simply represents selective growth of inherently drug-resistant clones present in the initial tumor (selection).

Although the *in vivo* study of molecular mechanisms leading to drug resistance is difficult, cell lines resistant to drugs such as doxorubicin, vinblastine, or colchicine have provided useful *in vitro* models (3). The cell lines selected for resistance against any one of these agents frequently display cross-resistance to other structurally dissimilar drugs, a phenomenon known as multidrug resistance (MDR). The MDR phenotype is accompanied by the expression of P-glycoprotein (P-gp), a membrane associated 170-kDa glycoprotein that is a product of the multidrug resistance 1 (*mdr1*) gene (also known as PGY1) (4-6). The MDR cell lines also display a complex spectrum of biochemical and cytogenetic alterations that may be primary or secondary to the development of drug resistance. Using the cDNA microarray technique, Kudoh *et al* (7) recently identified several genes in MCF-7 cells whose expression is acutely induced in response to doxorubicin treatment. Interestingly, a subset of these genes is constitutively overexpressed in doxorubicin-resistant cells, however, their functional significance in acquired or intrinsic drug resistance remains to be determined.

Our previous studies suggested that the expression of tissue transglutaminase (tTGase) (8), a Ca^{+2}-dependent protein crosslinking enzyme, is highly increased in drug-resistant MCF-7 (MCF-7/DOX) cells. The expression of this enzyme has been implicated in the onset of apoptosis in several cell-types (9,10). Thus, forced expression of tTGase could cause

increased rate of spontaneous apoptosis or rendered the cells highly susceptible to various death-inducing stimuli (11,12). These observations prompted our search for the factors that could contribute towards the ability of MCF-7/DOX cells to survive in otherwise toxic levels of tTGase. Our recent results suggested that MCF-7/DOX cells carry defective intracellular calcium pools, the feature that could render tTGase inactive and protect the MCF-7/DOX cells from its toxic effects (13). In this report, we describe some biochemical and cytogenetic features that are unique to MCF-7/DOX cells and may define the inherently drug-resistant subclones present in the starting drug-sensitive MCF-7/WT cells.

Materials and methods

Cell lines and tumor tissues. The parental human breast cancer MCF-7 cell line (MCF-7/WT) and prostate cancer cell line, LnCaP were obtained from the American Type Culture Collection (Rockville, MD). The 2 multidrug-resistant MCF-7 cell lines used in this study have been described previously (8,14). The MCF-7/WT/DOX cell line was established by continuous culture of MCF-7/WT cells in the presence of 1 μg/ml doxorubicin. Paraffin-embedded sections (5 μM thick) from 60 archived tumor specimens were obtained from untreated breast cancer patients. The samples were obtained from the M.D. Anderson Breast Tissue Bank following approval by the Institutional Committee for the Welfare of Human Subjects. Cell viability was assessed following appropriate treatment by microscopic examination of trypan blue stained cells or was measured using the standard MTT assay, as described (8). Cell-cycle analysis was performed by staining the cells with acridine orange (8 μg/ml) (Polysciences, Warrington, PA) in the presence of 0.1% Triton X-100 and then analyzing them on a Becton Dickinson FACScan flow cytometer. For each determination, a minimum of 20,000 cells were analyzed. The cells accumulating in the sub-G1 phase of cell cycle were considered apoptotic (15).

The RT-PCR for caspase-3 transcript was done as described (16). Briefly, total RNA from MCF-7 cell sublines and LNCaP cells was reverse transcribed and amplified by PCR using primers flanking the entire coding region of *CASP-3* mRNA. For cDNA sequencing, the PCR products were subcloned into pCR2.1 plasmid (Invitrogen) and sequenced in both directions by using an automated DNA sequencer (310 Genetic Analyzer, Applied Biosystems). For Western blot analysis, the following antibodies were used: caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA), PARP (Upstate Biotechnology, Lake Placid, NY), tTGase (CUB74; Neomarkers, Farmington, CA), P-gp (Dako, Carpinteria, CA), and ß-actin (Sigma Chemical Co., St. Louis, MO).

Chromosome preparation and G-banding. Cells were grown to approximately 75% confluency and treated with demecolcine (0.04 μg/ml) for 35-40 min at 37°C. The treated cells were dislodged, centrifuged, and exposed to a hypotonic solution (0.060 M KCl) for 20 min. The cells were then fixed in methanol-acetic acid mixtures (3:1, vol/vol) for 10 min, washed three times with the fixative, and dropped on glass slides for air-drying preparation. Optimally aged slides were treated for the induction of G-banding, following the routine procedures described elsewhere (17). From each cell line, 20-25 metaphase plates were photographed and at least 5 to 10 complete karyotypes were made for the identification of marker chromosomes. Altered chromosomes containing abnormally banded regions were identified from additional G-banded metaphase spreads.

Immunohistochemistry. Formalin-fixed paraffin-embedded breast tumor samples were stained with tTGase-specific monoclonal antibody (CUB 74, Neomarkers). Briefly, after antigen retrieval (in 10 mM citrate buffer, pH 6.0), the antigen antibody reaction was revealed using a Vectastain ABC kit (PK 6102; Vector Laboratories, Inc., Burlingame, CA) and developed with 3,3'-diaminobenzidine. Nuclei were counterstained with hematoxylin.

Results

Higher propensity of MCF-7/DOX cells to undergo apoptosis. The starting point for our studies was the observation that MCF-7/DOX cells were exquisitely sensitive to apoptotic stimuli. Thus, treatment with staurosporine (50 nM for 48 h) consistently exerted higher cytotoxicity against MCF-7/DOX cells when compared to the MCF-7/WT cells under similar conditions (Fig. 1A). Accordingly, a much pronounced DNA fragmentation was observed in MCF-7/DOX cells as revealed by accumulation of apoptotic sub-G1 phase cells in a fluorescence-activated cell sorter (FACS) analysis (Fig. 1B). On conventional agarose gel electrophoresis, a typical apoptotic DNA laddering was also observed in staurosporine-treated MCF-7/DOX cells (data not shown). The staurosporine-induced accumulation of apoptotic MCF-7/DOX cells was time-dependent (Fig. 1C). Under similar conditions the MCF-7/WT cells, however, showed no noticeable increase in sub-G1 apoptotic cell number (Fig. 1C).

One major difference between MCF-7/WT and MCF-7/DOX cells is that the latter express high levels of a pro-apoptotic enzyme, tTGase (8). Therefore, we next determined whether increased propensity of MCF-7/DOX cells to undergo apoptosis was associated with increased apoptotic signaling events. We started by comparing down stream signaling events induced in response to staurosporine treatment in MCF-7/WT and MCF-7/DOX cells. For example, poly(ADP-ribose)polymerase (PARP), which is thought to be a part of the cascade signaling for DNA damage in the cells (18), was consistently cleaved to its apoptotic 24- and 89-kDa fragments in MCF-7/DOX cells treated with staurosporine (Fig. 2A). No such cleavage of PARP was observed in MCF-7/WT cells, confirming that MCF-7/WT cells respond differently to staurosporine treatment than do the MCF-7/DOX cells.

Full-length functional caspase-3 in MCF-7/DOX cells. In vitro, PARP can be cleaved by almost any of the known caspases, including caspase-1; *in vivo,* however, PARP is processed only by caspase-3 or -7 (19). Because the MCF-7/WT cells have been shown to lack caspase-3 expression as a result of a functional deletion within exon 3 of the *CASP-3* gene (16), it was tempting to determine the pathway that leads to the processing of PARP in MCF-7/DOX cells. We examined caspase-3 levels in both MCF-7/WT and MCF-7/DOX cells before and after staurosporine treatment. As anticipated, the

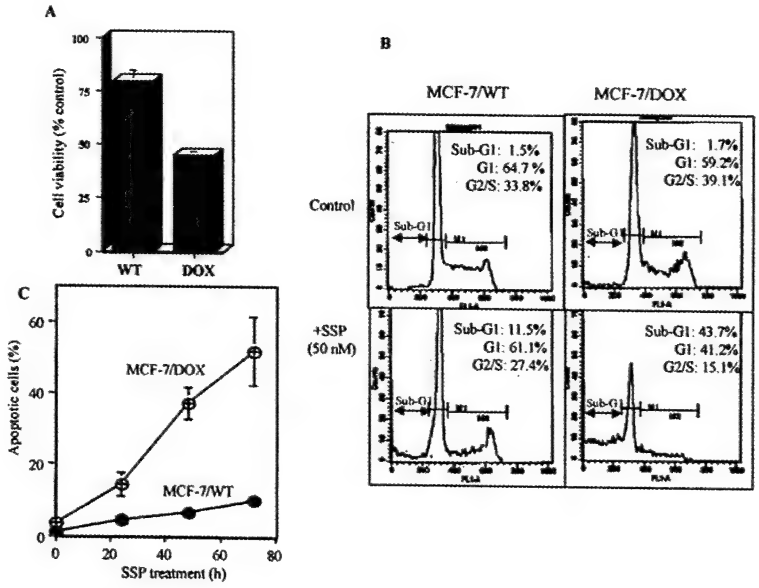

Figure 1. Staurosporin (SSP)-induced cell-death in drug-sensitive (MCF-7/WT) and doxorubicin-resistant (MCF-7/DOX) human breast carcinoma (MCF-7) cells. (A), SSP-treated (50 nM for 48 h) MCF-7 cells were subjected to MTT assay and the cell viability was determined as described in Materials and methods. (B), SSP-untreated and treated MCF-7 cells were subjected to flow cytometry analysis after staining with acridine orange. Plot showing DNA content of cells incubated for 48 h in medium alone (top panels) or medium containing SSP (bottom panels). Cells that exhibit the sub-G1 DNA levels are considered apoptotic. (C), Time-dependent increase in SSP-induced apoptosis of MCF-7/DOX and MCF-7/WT cells. The data shown are representations of three separate experiments.

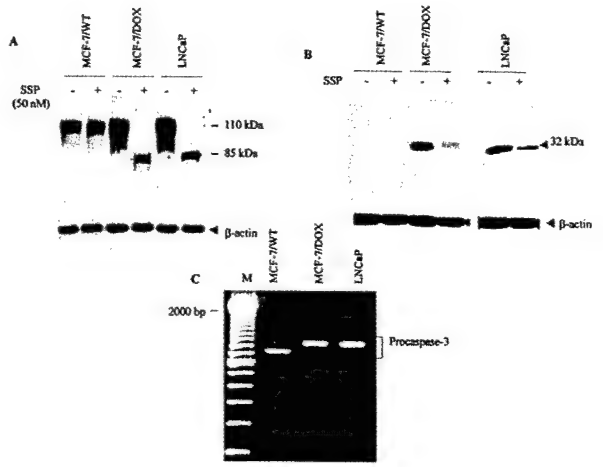

Figure 2. Expression and activation of caspase-3 in MCF-7/DOX cells. The drug-sensitive and -resistant MCF-7 cells were incubated in medium alone (-) or medium containing 50 nM staurosporine (SSP) (+). Forty-eight hours later, the cells were lysed and analyzed for poly(ADP-ribose)polymerase (PARP) (A) or caspase-3 (B) by Western blot analysis by using either PARP-specific (1:2000 dilution; Upstate Biotechnology) or caspase-3-specific (1000 dilution; Pharmingen) antibody. The membranes were re-probed with a mouse monoclonal anti-ß-actin antibody (Sigma; 1:4000 dilution) to determine the protein loading. The Amersham-Pharmacia ECL system was used to detect antigen/antibody reaction. The prostate cancer cell line, LNCaP was used as a positive control. (C), RT-PCR analysis of caspase-3 mRNA in MCF-7/DOX, MCF-7/WT, and LNCaP cells by using caspase-3-specific primers, flanking the entire coding region of caspase-3. Lane M is the 100-bp ladder.

MCF-7/WT cells lacked procaspase-3 protein expression, as determined by Western blot analysis. Surprisingly, the MCF-7/DOX cells that are derived from MCF-7/WT cells by culturing them in the continuous presence of increasing doxorubicin concentrations (14), exhibited a major immunoreactive band corresponding to procaspase-3 protein (Fig. 2B). The treatment of MCF-7/DOX cells with staurosporine yielded active fragments of caspase-3, as determined by a decrease or disappearance of the procaspase 32-kDa band (Fig. 2B). To further assess this observation, we studied caspase-3 mRNA expression in MCF-7/WT and MCF-7/DOX cells by reverse transcription-polymerase chain reaction (RT-PCR). Using the caspase-3-specific primers flanking the entire coding region of the *CASP-3* gene, MCF-7/WT cells gave rise to a ~750-bp fragment, which was shorter than the full-length caspase-3 transcript (834-bp) amplified from the control LNCaP prostate cancer cell line (Fig. 2C). Like the LNCaP cells, MCF-7/DOX cells gave rise to a full-length caspase-3 transcript (Fig. 2C).

We further investigated the discrepancy between the levels of caspase-3 expression in the parental MCF-7/WT cell line and in its subline, MCF-7/DOX. The PCR products from the two cell lines were subcloned into a pCR-2.1 cloning vector and sequenced in both directions by using an automated DNA sequencer. The cDNA sequence of caspase-3 from MCF-7/WT cells revealed a 125-bp deletion from position 54 in Pro18 to 178 in Gly60 (data not shown). As previously reported (16) the shift in the open reading frame resulting from this deletion is responsible for the loss of procaspase-3 protein expression in MCF-7/WT cells. Interestingly, the cDNA sequence of caspase-3 from MCF-7/DOX cells was identical to the full-length cDNA sequence of caspase-3 obtained from the control LNCaP cells (data not shown) and to the earlier reported YAMA/CPP32 cDNA sequence (20). To further resolve this issue, we compared the chromosomal profiles of the MCF-7/WT and MCF-7/DOX cell lines in detail.

Different chromosomal profiles of MCF-7/WT and MCF-7/DOX cells. The chromosome numbers in cells of the parental

Figure 7. tTGase expression in breast cancer cells. Representative sections from 60 archived samples obtained from untreated breast cancer patients are shown. Though majority of the samples (70%) contained no detectable tTGase (a and b), 9% samples showed focal areas of intense tTGase staining (c and d). Other 15% samples exhibited small islands of tTGase-positive cancer cells (e and f), and the remainder 6% samples showed diffused tTGase expression throughout the tumor areas (g and h). The immunoreactivity of anti-tTGase antibody was quite selective. The endothelial cells for example, which contain high levels of tTGase, invariably showed strong immunoreativity in the tumor vasculature (a and b). Similarly, control immunoglobulin G showed no immunostaining in the tumor cells or the stromal cells (data not shown).

MCF-7/DOX cells is a result of expansion and propagation of an inherently resistant subclone that is present in the starting population of MCF-7 cells.

Discussion

The emergence of resistance to antineoplastic drugs has been

length functional caspase-3, expression of tTGase and P-gp, and several karyotypic alterations. The most notable chromosomal alterations included the absence of a normal copy of chromosome 1, the presence of two or three UM chromosomes containing abnormally banded regions, the presence of a derivative chromosome 12 with translocation on its long arm of chromosome 3, and the presence of one normal copy and one altered copy of the X chromosome (Fig. 3B). The altered X chromosome in MCF-7/WT cells had no similarity to the modified X chromosome in MCF-7/DOX cells. Based on these differences, we initially concluded that MCF-7/WT and MCF-7/DOX cell lines are quite different from each other and may not represent a common ancestor. In this context, it is interesting to note that based on the DNA fingerprinting analysis it was recently concluded that MCF-7/DOX cells are not related to MCF-7/WT cells and that the two cell lines were derived from two different donors (23). More recently, using another drug-resistant MCF-7 cell line (MCF-7 TH) that was independently selected for resistance against doxorubicin, Pirnia *et al* (24) raised a similar alert. Similar to our findings (Fig. 2), these authors observed that despite a complete loss of caspase-3 protein in the parental MCF-7 cells, MCF-7 TH cells expressed full-length functional caspase-3 protein. On the basis of these observations, these authors concluded that even MCF-7 TH cell line is not related to MCF-7 cells. Our results, presented in this report help to clarify this discrepancy and demonstrated that drug-resistant MCF-7 cells indeed represent a subclone of the MCF-7/WT cells with striking differences in their genotypic and phenotypic features. Since the two cell lines exhibit significant difference in their karyotypes (Fig. 3), it is likely that they could yield different DNA fingerprinting and restriction fragment length polymorphism (RFLP) maps, as observed earlier by other groups (23).

Furthermore, it is difficult to reconcile with the observation that treatment of MCF-7/WT cells, which harbor truncated *CASP-3* gene, with doxorubicin (1 μg/ml) led to the growth of a subclone that contained full-length functional *CASP-3* gene. These findings could be explained only on the premise that MCF-7/WT cells have at least two subpopulations: the predominant one, which contains the mutated *CASP-3* gene; and the minor clone, which harbors the full-length *CASP-3* gene. Similar to the caspases-3, the cells that survived cytotoxic effects of the drug expressed high levels of another proapoptotic protein, tTGase but lacked the expression of an anti-apoptotic protein, Bcl-2 (unpublished data). In view of these findings, it will be interesting to determine the mechanisms that underlie the resistance of MCF-7/DOX cells to chemotherapeutic drugs, despite their high propensity to apoptotic stimuli. It is likely that overexpression of proteins, like P-gp may play predominant role in drug resistance. Whether increased expression of P-gp and tTGase, similar to other genetic alterations, represent changes that are present from the beginning in MDR^+-subclones of the breast tumor cells or are induced in response to drug treatment remains to be determined. However, there is some evidence to support the argument that at least P-gp expression can be acutely induced in human cancer cells following their exposure to doxorubicin (25,26). Moreover, continuous culture of MCF-7/DOX cells in doxorubicin-free medium could partially revert the sensitivity of these cells to the toxic effects of the drug, the phenomenon that was associated with a time-dependent decrease in P-gp expression. Despite complete loss of P-gp, the revertant cells continue to express tTGase at levels comparable to those in the parental MCF-7/DOX cells (13). Moreover, we did not detect any significant level of tTGase induction in MCF-7/WT cells following their treatment with doxorubicin. These results suggest that P-gp represents an inducible gene, whereas tTGase expression may represent an inherent property of drug-resistant cancer cells. In view of this, the presence of tTGase-positive cancer cells in untreated breast cancer patients may support the contention that tTGase expression may represent inherently resistant tumor subclones. Further studies to correlate the presence of tTGase and the refractoriness of the cancer cells to adriamycin-based chemotherapy, are currently in progress in our laboratory.

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ORIGINAL PAPERS

Down-regulation of caspase 3 in breast cancer: a possible mechanism for chemoresistance

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Caspase-3 is a member of the cysteine protease family, which plays a crucial role in apoptotic pathways by cleaving a variety of key cellular proteins. Caspase-3 can be activated by diverse death-inducing signals, including the chemotherapeutic agents. The purpose of this study was to determine the levels of caspase-3 expression in breast tumor samples and to determine whether alterations in its expression can affect their ability to undergo apoptosis. Primary breast tumor and normal breast parenchyma samples were obtained from patients undergoing breast surgery and the expression of caspases-3 was studied. Similarly, normal mammary epithelial cells and several established mammary cancer cell lines were studied for caspases-3 expression by reverse transcriptase-polymerase chain reaction, Northern blot analysis, and Western blot analysis. Approximately 75% of the tumor as well as morphologically normal peritumoral tissue samples lacked the caspase-3 transcript and caspase-3 protein expression. In addition, the caspases-3 mRNA levels in commercially available total RNA samples from breast, ovarian, and cervical tumors were either undetectable (breast and cervical) or substantially decreased (ovarian). Despite the complete loss of caspase-3, the expression levels of other caspases, such as caspase-8 and caspase-9, were normal in all of the tumor samples studied. The sensitivity of caspase-3-deficient breast cancer (MCF-7) cells to undergo apoptosis in response to doxorubicin and other apoptotic stimuli could be augmented by reconstituting caspase-3 expression. These results suggest that the loss of caspases-3 expression may represent an important cell survival mechanism in breast cancer patients.
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Introduction

Apoptosis is a genetically regulated form of cell death that plays an important role in eliminating infected, damaged, and other unwanted cells from the body (Kerr *et al.*, 1972). With the realization that defects in apoptosis can contribute to diseases like cancer, interest in the control of apoptosis has grown exponentially among cancer researchers. Apoptosis can be triggered by various extracellular and intracellular stimuli that result in coordinated activation of a family of cysteine proteases called caspases.

About 14 caspases have been described so far in mammalian systems (Evan and Littlewood, 1998; Johnstone *et al.*, 2002; Eckhart *et al.*, 2000; Ahmad *et al.*, 1998). On the basis of their role in apoptosis, caspases can be categorized into two major subgroups. The upstream or initiator caspases (e.g., caspase-8, -9 and -10) are activated by apoptotic signals, resulting in the activation of the downstream or executioner caspases (Nicholson, 1999). Following their activation, the executioner caspases, which include caspase-3, -6 and -7, catalyze the specific cleavage of many key cellular proteins, such as poly(ADP-ribose) polymerase, inhibitor of caspase-activated DNase, gelsolin, 4-GDI, α- and β-fodrin, and epidermal growth factor receptor (Thorneberry and Lazebnik, 1998; Cryns and Yuan, 1998). The cleavage of these proteins results in membrane blebbing, chromatin condensation, and DNA fragmentation, the hallmark changes associated with apoptosis. A mitochondrial flavoprotein, apoptosis-inducing factor (AIF), can also induce morphological apoptosis in caspase-independent manner in response to certain apoptotic stimuli (Cande *et al.*, 2002). Nevertheless, caspase-3 (also known as CPP32, Yama, and apopain) is considered to be the central protein in the execution of apoptosis (Enari *et al.*, 1996) and to play a pivotal role in the development of the central nervous system. For example, caspase-3 knockout mice exhibited decreased apoptosis in the brain and died prematurely (Kuida *et al.*, 1996).

Genetic or epigenetic alterations that disrupt the ability of cells to undergo apoptosis can lead to the

development of cancer. For example, *p53* is frequently mutated in aggressive and in chemoresistant tumors. The expression of *Apaf-1*, a cell-death effector that acts with cytochrome-*c* and caspase-9 to mediate p53-dependent apoptosis is lost in metastatic melanomas (Soengas *et al.*, 2001). Similarly, the gene for the effector caspase-8 is frequently inactivated in childhood neuroblastomas (Teitz *et al.*, 2000). Moreover, resistance to apoptotic stimuli has been reported frequently in MCF-7 human breast carcinoma cells that lack expression of caspase-3 as a result of a 47 bp deletion in exon 3 of the *CASP3* gene (Janicke *et al.*, 1998; Yang *et al.*, 2001).

Considering the central role caspase-3 plays in executing apoptosis and the observation that several established breast cancer cell lines exhibit altered caspases-3 expression, we examined the expression of caspase-3 in freshly isolated normal and malignant breast tissue samples. Our results revealed some interesting findings; specifically, approximately 75% of the breast tumor samples lacked the caspase-3 transcript and expression of caspase-3 protein, while the remaining samples showed substantial decreases in caspase-3 expression. More interestingly, a similar loss in caspase-3 expression was evident in morphologically normal peritumoral tissue samples obtained from breast cancer patients. These results suggest that loss of caspase 3 expression may represent an important mechanism of cell survival and chemoresistance by breast cancer cells.

Results

Caspase-3 expression in breast cancer cell lines

As our first step in determining the levels of caspase-3 in breast cancer cells, we performed Western blot analysis on a panel of human mammary cancer cell lines (Figure 1). Most of the cell lines that we studied contained abundant levels of caspase-3 protein. The three normal human breast epithelial cell lines (MCF10A and the two finite-life-span epithelial cell lines obtained from Clonetics, San Diego, CA, USA) showed considerable levels of caspase-3 protein expression. However, as previously reported (Janicke *et al.*, 1998), MCF-7 cells completely lacked caspase-3 protein expression (Figure 1). Interestingly, two other breast cancer cell lines, BT-20T and ZR-75T, also showed a complete lack of caspase-3 protein expression (Figure 1). Using caspase-3-specific primers, RT–PCR analysis of these two cell lines demonstrated a truncated (approximately 750 bp) transcript that upon sequencing revealed a 125 bp deletion from position 54 in Pro18 to position 178 in Gly60 (unpublished observations). As previously observed (Jenicke *et al.*, 1998), the shift in the open reading frame resulting from this deletion may account for the loss of procaspase-3 protein in BT-20T and ZR-75T cells. Western blot analysis also revealed that the levels of caspases-3 protein expression in some other breast cancer cell lines (Hs854T, HCC1428, BT549, and BT483) were on average five to seven times lower than those in the NME cells (Figure 1).

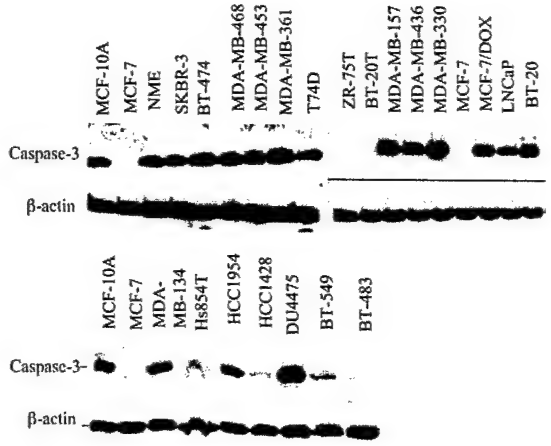

Figure 1 Levels of caspase-3 protein expression in various breast cancer cell lines were detected using Western blot analysis. Sixty micrograms of sample protein was applied in each lane and fractionated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and probed with an anti-capsase-3 antibody as described in Materials and methods. The membranes were stripped and reprobed with β-actin antibody to ensure even loading

Expression of caspase-3 in human breast cancer

In view of the various caspase-3 protein expression levels and transcript sizes in different breast cancer cell lines, we next determined whether similar variations could occur in human breast cancer cells. Breast tissue specimens were collected from a total of 46 patients, including 31 with adenocarcinoma (four of whom had received preoperative chemotherapy), ten with a history of breast cancer, three with benign breast disease, and two undergoing breast reduction surgery. Clinical and pathologic data, including TNM stage, hormone receptor (estrogen receptor (ER) and progesterone receptor (PR)) status, and Her-2 expression levels from the 31 patients with adenocarcinoma are shown in Table 1. Total RNA was extracted from benign and malignant tissue samples, and RT–PCR using caspase-3-specific primers was performed. The results shown in Figure 2a, and Table 1 revealed that about 75% of the breast tumor samples lacked caspase-3 transcript; the remaining 25% had detectable expression levels of caspase-3 mRNA, albeit the levels were significantly lower than those observed in the NME cells.

Morphologically normal breast parenchyma samples obtained from the vicinity of the tumor tissue showed similar lack of or reduction in caspase-3 expression (Figure 2b). Out of the 12 such samples studied (from patients #1–12; Table 1), only one demonstrated detectable caspase-3 expression by RT–PCR analysis. On the other hand, a substantial number (seven out of 10) of normal tissue samples obtained from patients who had a history of breast cancer and had undergone

Table 1 Clinical features of breast cancer patients studied for caspase-3 expression

Patient # no.	*Patient age, years*	*TNM stage*	*Hormone receptor status*		*ki-67*	*Expression of Her-2*	*Casp-3*
			ER	*PR*			
Breast carcinoma							
1	59	T1N0M0	–	–	High	+	–
2[e]	46	T4N0M0	–	–	High	–	–
3	68	T2N1M0	–	–	High	–	–
4	46	T1N0M0	+	+	Low	+	–
5	49	T1N0M0	+	–	Moderate	ND	–
6	72	T2	+	+	N/A	–	–
7[e]	56	T4N0M0	–	–	Moderate	–	–
8	52	T3N1M0	–	–	Moderate	–	–
9	65	T0N0M0	N/A	N/A	N/A	N/A	–
10	45	T2N1M0	–	–	High	–	–
11	64	T2N0M0	+	+	Low	–	+
12	50	T3N1M0	+	–	High	–	–
13	52	T2N1M0	–	–	High	–	–
14	74	T1N0M0	+	+	Low	–	+
15	59	T0N0M0	N/A	N/A	N/A	N/A	+
16	40	T2N2M0	+	+	Low	–	–
17[e]	56	T4N1M1	–	–	High	–	+
18	52	T1N0M0	+	+	Low	–	–
19	68	T1N0M0	+	+	Low	–	–
20[e]	48	T2N1M0	–	–	High	+	–
21	56	T2N0M0	–	–	High	–	–
22	53	T2N1M0	–	–	High	–	–
23	54	T2N0M0	+	+	Low	–	+
24	87	T2N0M0	–	–	High	–	–
25	51	T2N0M0	–	–	Moderate	–	–
26*	60	T2N0M0	+	+	High	–	–
27	59	T1N0M0	+	+	Low	–	–
28	59	T1N1M0	+	+	Low	–	+
29	56	T1N0M0	+	+	Moderate	–	–
30	58	T4N2M0	+	–	High	–	+
31	38	T1N0M0	+	+	Low	–	+
Morphologically normal breast parenchyma (from patients with breast cancer)[a]							
1	59						–
2	46						–
3	68						–
4	46						–
5	49						–
6	72						–
7	56						–
8	52						–
9	65						–
10	45						+
11	64						–
12	50						–
(from patients with cancer history)[b]							
32	81						–
33	64						–
34	43						+
35	45						+
36	66						+
37	57						+
38	56						+
39	55						+
40	53						+
41	43						–
(from patients with non-neoplastic disease)[c]							
42	55	Hyperplasia					+
43	66	Hyperplasia					+
44	25	Fibrocystic					+
45	51	Breast reduction					+
46	36	Breast reduction					+
4*	46	Hyperplasia					+

[a]Morphologically normal breast parenchyma samples were obtained from the patients #1–12 and tested for caspase-3 expression. [b]Morphologically normal breast parenchyma samples were obtained from patients who had undergone tumor resection surgery in the past. [c]Normal breast tissue samples were obtained from individuals with no history of neoplastic disease. [d]Patient had received chemotherapy prior to the surgery. [e]Tissue sample from the normal side of the breast of patient #4. TNM = tumor, node, metastasis; ND = not done; N/A = not applicable

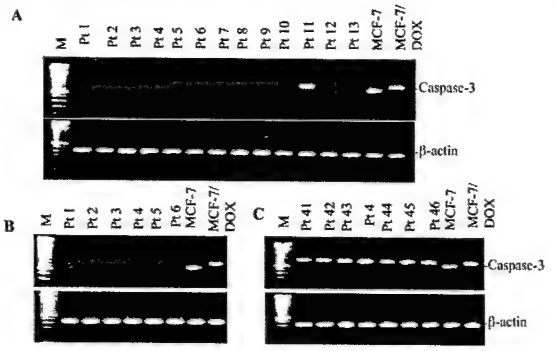

Figure 2 RT–PCR analysis of messenger RNA (mRNA) using primers specific for caspase-3 in breast tumor (**a**), adjacent normal (**b**), and normal mammary (**c**) tissue samples. Drug-sensitive (MCF-7) and drug-resistant (MCF-7/DOX) MCF-7 cells were used as positive controls for amplification of the truncated and full-length caspase-3, respectively. Amplification of β-actin was serially analysed for all of the samples to normalize for mRNA integrity and equivalent loading. M represents the 0.1 kb DNA ladder and Pt denotes patient

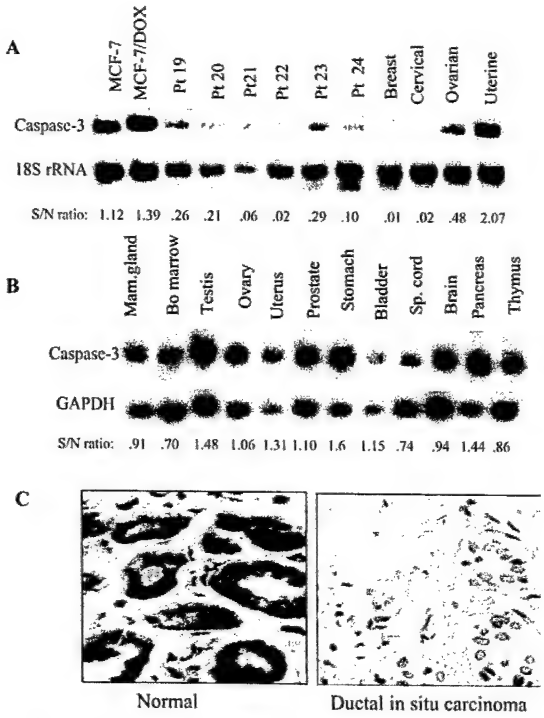

Figure 3 Caspase-3 gene expression in human mammary carcinoma (**a**) and normal tissue samples (**b**) as determined by Northern blot analysis (**a** and **b**) or *in situ* RT–PCR (**c**). Data for wild-type MCF-7 and drug-resistant MCF-7/DOX breast cancer cell lines, six representative breast carcinomas, and total RNA isolated from human breast, cervical, ovarian, and uterine tumor samples are shown. (**b**) shows the caspase-3 mRNA expression in various normal human tissues obtained via probing a 12-lane Multiple tissue Northern blot. The S/N ratios indicate the ratio between caspases-3 band and 18S ribosomal RNA (**a**) or GAPDH (**b**) band. Pt denotes patient

surgical removal of their tumors in the past (patients #32–41; Table 1) showed detectable levels of caspases-3 expression. As a control, we used the normal breast tissue samples obtained from the patients undergoing breast reduction surgery (patients #45 and 46; Table 1) or from patients with benign breast disease (patients #42–44 and second breast from patient #4; Table 1). All the five normal breast tissue samples showed strong levels of caspase-3 transcript expression (Table 1; Figure 2c).

The loss of caspase-3 expression in breast cancer cells was further confirmed by Northern blot analysis. The results shown in Figure 3, clearly demonstrated that caspase-3 mRNA expression levels in breast tumors were at least 10–50 times lower than those in normal breast tissue or breast cancer cell lines. Specifically, MCF-7 cells showed truncated caspase-3 mRNA due to a 125 bp deletion in the transcript (Janicke *et al.*, 1998). Nevertheless, MCF-7/DOX cells that were derived via continuous culture of MCF-7 cells in the presence of doxorubicin, exhibited a full-length caspase-3 transcript (Figure 3a) as previously demonstrated (Pirina *et al.*, 2000; Devrajan *et al.*, 2002). We also tested the commercially available total RNA samples that had been isolated from the human breast, cervical, uterine, and ovarian tumors (Clontech Laboratories). To our surprise, the RNA samples isolated from not only the breast tumor sample but also cervical tumor samples, lacked detectable caspase 3 mRNA levels (Figure 3a). The RNA sample from ovarian tumors contained approximately four times lower caspase 3 mRNA than the uterine tumor sample, which was the only tumor sample that we tested, exhibited substantial levels of the caspase-3 transcript. However, a strong signal for caspase-3 mRNA was detected in various normal tissue samples, including the mammary gland, when the human Multiple Tissue Northern blot was probed under identical conditions (Figure 3b). The status of caspase-3 transcript expression in breast tissue samples was further determined by *in situ* RT–PCR analysis, using paraffin-embedded tissue sections. Figure 3b shows the caspase-3 transcript expression in representative breast tissue samples from a normal and a cancer patient. The *in situ* RT–PCR analysis further confirmed the deficient status of caspase-3 transcript expression in breast tumor. The caspase-3 expression in normal breast sample was restricted mainly to the ductal epithelial and myoepetelial cells (Figure 3b).

Next, we determined the caspase-3 protein levels in breast cancer samples by using immunoblot analysis. No immunoreactive band corresponding to the authentic procaspase-3 protein (32 kDa) was evident in any of the six breast tissue samples (Figure 4A) that were negative for caspase-3 expression according to RT–PCR analysis (Table 1). Under similar conditions, however, the cell lysates from a prostate cancer LNCaP cell line that we used as a positive control, showed a strong immunoreactive band corresponding with procaspase-3 protein (Figure 4a). Similarly, the extracts from breast tissue samples that exhibited caspase-3

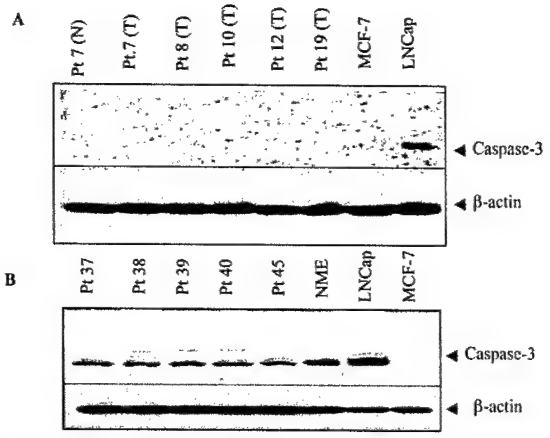

Figure 4 Western blot analysis of breast tissue samples for caspase-3 protein expression. Breast carcinoma (T) or adjacent normal (N) tissue samples obtained from breast cancer patients were homogenized in buffer, and 60 μg of homogenate protein was separated on an sodium dodecyl sulphate polyacrylamide gel electrophoresis. The membranes were probed with an anti-caspase-3 antibody. (**a**) Breast tissue samples that showed no detectable caspases-3 transcript levels by RT–PCR analysis (Table 1). (**b**) Breast tissue samples that showed detectable caspase-3 transcript levels. The finite-life-span NME and LNCap cells showed a strong 32 kDa immunoreactive band corresponding with caspase-3 protein. Wild-type MCF-7 cells completely lacked caspase-3 protein expression. The membranes were stripped and reprobed with anti-β actin antibody to confirm equal protein loading and even transfer. Pt denotes patient, (N) denotes adjacent normal tissue sample, (T) denotes tumor sample, and NME denotes normal mammary epithelial cells

transcript expression by RT–PCR (Table 1), also showed a 32 kDa immunoreactive band, corresponding to procaspase-3 protein (Figure 4b). A similar 32 kDa band was detected in extracts from the NME cells but MCF-7 cells completely lacked its expression, probably due to the 125 bp deletion in the transcript.

Effect of caspase-3 reconstitution on apoptosis

In order to determine the significance caspase-3 downregulation in breast cancer, we compared the ability of caspase-3-deficient and caspase-3 reconstituted MCF-7 cells to undergo apoptosis in response to calcium ionophore A23187 and staurosporine treatment. To reconstitute caspase-3, MCF-7 cells were transfected with pBabe/puro retroviral vector plasmid encoding a full-length procaspase 3 cDNA (pBS-CPP32) or empty vector (pBS) (Figure 5a). Flow cytometry analysis of ionophore A23187 (2 μM, 48 h) and staurosporine (50 nM, 48 h)-treated MCF-7 cells revealed significant apoptosis (accumulation in sub-G1 phase) in pBS-CPP32-transfected MCF-7 cells; MCF-7/pBS control cells under similar conditions showed no appreciable apoptosis (Figure 5b,c). Similarly, the multidrug resistant MCF-7/DOX cells which contain constitutive expression of caspase-3 protein (Devarajan *et al*., 2002), showed comparable apoptosis to pBS-CPP32 transfected MCF-7 cells in response to A23187 (Figure 5b) or staurosporine treatment (Figure 5c). Moreover, the treatment of MCF-7/DOX and MCF-7/pBS-CPP32 cells with ionophore A23187 yielded active fragments of caspase-3, as determined by a decrease or disappearance of the procaspase-3 32 kDa band by Western blot (data not shown). As previously reported (Yang *et al*., 2001), the MCF7/pBS-CPP32 cells exhibited at least 2–3-fold higher sensitivity towards doxorubicin-induced killing effect when compared with the control MCF-7/pBS cells (data not shown). These results suggested that lack of caspase-3 could render breast cancer cells resistant to doxorubicin as well as to other apoptotic stimuli.

Finally, to determine whether the loss of caspase-3 expression represented a selective lesion in breast cancer patients, we tested some caspase-3-negative tumor samples for the expression of other caspases. The results shown in Figure 6 demonstrated that, despite a complete lack of procaspase-3 expression, the breast tumors had high levels of procaspase-9 and -8 expression. These results suggested that the loss of caspase-3 expression represents a selective event in breast cancer cells.

Discussion

It is now becoming apparent that many drugs can kill tumor cells by activating common apoptotic pathways (Hickman, 1996; Clynes *et al*., 1998). Thus, somatic, genetic or epigenetic alterations that disable apoptosis can produce multidrug resistance. In this study, we demonstrate one such defect in breast cancer cells that can attenuate drug-induced apoptosis. A significant majority of the human breast tumors tested lacked caspase-3 expression. Caspase-3 is a member of the cysteine protease family, which plays a central role in the execution of apoptosis. We studied caspase-3 expression in breast cancer cells partly because of its important role in apoptosis but more so because in several of the breast cancer cell lines that we studied it was either altered or lost (Figure 1). Additionally, caspase-3 activation has been detected in response to a variety of apoptotic stimuli, including chemotherapeutic agents, irradiation, and cytokines (Salvesen and Dixit, 1997). Conversely, selective inhibition of caspase-3 has been associated with inhibition of cell death (Hasegawa *et al*., 1996; Silke *et al*., 2001). These observations imply that loss of caspase-3 expression may render breast cancer cells resistant to chemotherapy and radiation therapy. Indeed, MCF-7 cells, which lack caspase-3 expression as a result of a functional deletion mutation in the *CASP3* gene, are relatively insensitive to cisplatin, doxorubicin and etoposide. However, reconstitution with caspase-3 rendered MCF-7 cells sensitive to these and various other apoptotic stimuli (Figure 6) (Yang *et al*., 2001). Conversely, despite the lack of caspase-3 expression, MCF-7 cells undergo morphological apoptosis in response to a variety of agents (Eck-Enriquez *et al*., 2000). It is likely that these agents can induce apoptosis

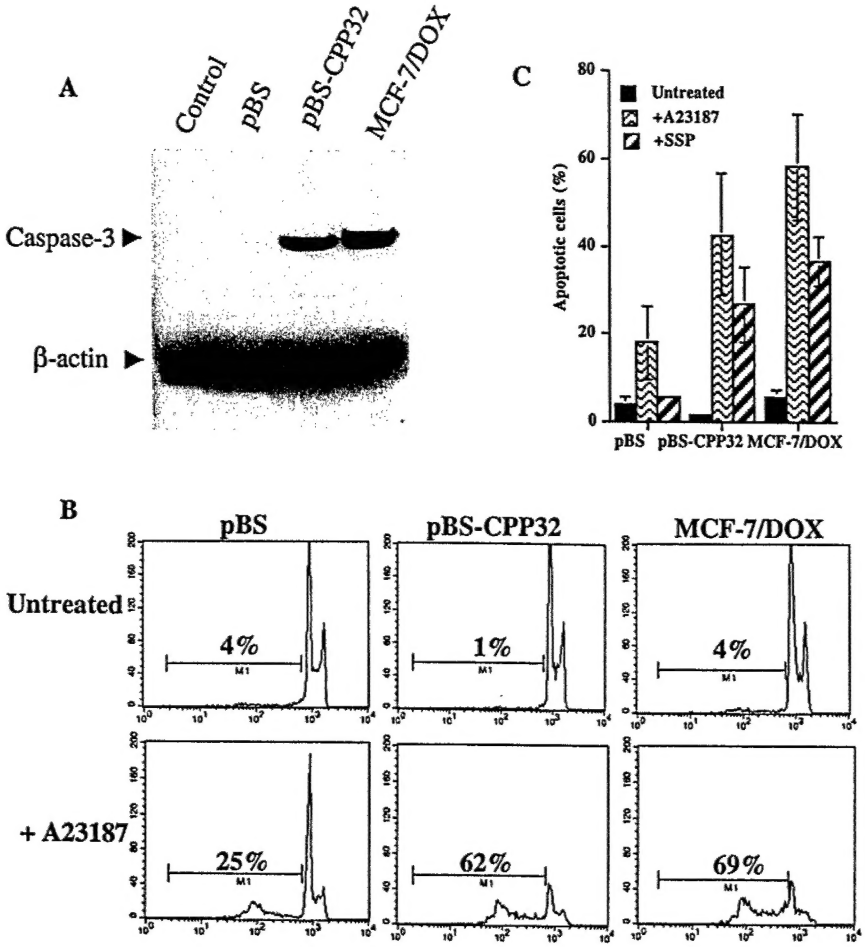

Figure 5 The effect of caspase-3 reconstitution on ionophore A23187 and SSP-induced apoptosis in MCF-7 cells. (**a**) Protein levels of caspase-3 in parental MCF-7 cells and vector-alone (pBS) or caspase-3 (p-CPP32)-transfected MCF-7 cells were detected by Western blot. Drug-resistant MCF-7 (DOX) cells served as a positive control for caspase-3 expression. (**b**) Untreated and ionophore A23187 (2 μM)-treated MCF-7 cells were subjected to flow cytometry analysis after staining with acridine orange. Plot showing DNA content of cells incubated for 48 h in medium alone (top panel) or medium containing ionophore (bottom panel). Cells that exhibit the sub-G1 DNA levels are considered apoptotic. (**c**) Following the incubation in medium alone (untreated) or medium containing ionophore A23187 (2 μM) or SSP (50 nM), cells were subjected to flow cytometry analysis. The mean $\pm$ s.d. of percent apoptotic cells that exhibited sub-G1 DNA levels after 48 h of treatment are shown

either in a caspase-independent manner (for example via AIF; Cande *et al.*, 2002) or may involve alternative downstream caspases, such as caspase-6 and -7 (Liang *et al.*, 2001). Nevertheless, drug-resistant breast cancer cells could be rendered sensitive to epirubin, taxol, and ectoposide simply by overexpressing the caspase-3 protein (Friedrich *et al.*, 2001).

Another interesting aspect of this study was the observation that caspase-3 expression is also lost in normal looking breast parenchyma from breast cancer patients. Out of the twelve patients from whom such samples were obtained in this study, only one (patient #10) showed detectable levels of caspase-3 expression by RT–PCR analysis (Table 1). In contrast, seven (patients #34–40) out of ten normal breast parenchyma tissue samples studied from patients who had undergone surgical tumor resection, showed normal levels of caspases-3 expression. The two normal breast tissue samples obtained during breast reduction surgery (patients #45 and 46) showed normal caspase-3 expression levels by RT–PCR (Figure 2c) and immunoblotting (Figure 4b). Interestingly, the normal contralateral breast tissue sample from one patient (patient #4) exhibited a detectable level of caspase-3 transcript (Table 1); whereas both the tumor sample and the adjacent normal parenchyma sample from the same patient but obtained from the tumor-affected side showed a complete loss of caspases-3 expression. Moreover, substantial caspase-3 expression levels were observed in breast tissue samples obtained from three individuals (patients #42–44) who did not have neoplastic breast disease.

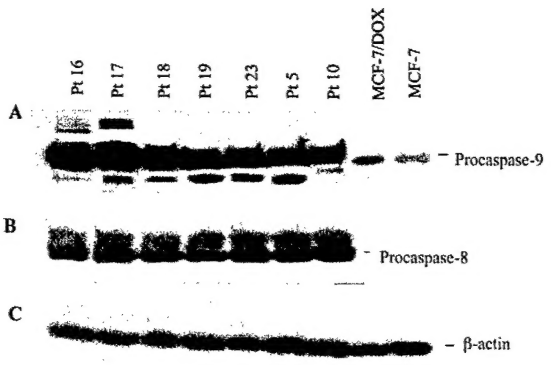

Figure 6 Western blot analysis showing caspase-8 and -9 protein expression in caspase-3-negative breast tumor samples. Sixty micrograms of homogenate protein was separated on sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Each membrane was probed with specific antibodies against caspase-9 (a) or caspase-8 (b). One of the membranes (a) was stripped and reprobed with anti-β actin antibody to confirm equal protein loading and even transfer (c). Pt denotes patient

These results suggest that a lack of caspase-3 can attenuate apoptosis in response to certain stimuli, including chemotherapeutic drugs. Disruption of the intrinsic apoptotic pathways is frequently observed in cancer cells. For example, the tumor suppressor gene *p53* is inactivated in more than 50% of human cancers, resulting in the removal of a key component of the DNA damage sensor that can induce the apoptotic effector cascade (Harris, 1996). Conversely, the proteins that are known to serve as antiapoptotic survival signals, such as *bcl-2, bcl-x, ras,* and c-*abl,* are often overexpressed in tumor cells and cell lines (Martin and Green, 1996). The correction of these alterations is associated with an increased propensity for the tumor cells to undergo apoptosis and re-establishes chemosensitivity (Johnstone *et al*., 2002; Wallace-Brodeur and Lowe, 1999).

Importantly, the ability of cells to evade apoptosis is one of the essential hallmarks of cancer cells, the skill that can help them breach the anticancer defense mechanisms (Hanahan and Weinberg, 2000). Thus, loss of expression of caspase-3 and/or other caspases may serve as a pivotal step in the survival of mutated somatic cells. Unlike that of caspase-3, the expression of caspase-8 and -9 was normal in breast tumor samples in the present study (Figure 6). However, complete inactivation of the gene for caspase-8 was recently reported in neuroblastomas, a childhood tumor of the peripheral nervous system (Teitz *et al*., 2000). Additionally, caspase-8-null neuroblastoma cells were resistant to doxorubicin- and death receptor-induced apoptosis. Furthermore, more than 80% of prostate tumors exhibit a complete lack of caspase-1 protein expression and reduced levels of caspase-3 expression (Winter *et al*., 2001). Similarly, *Apaf-1*, a cell-death effector protein that acts in association with cytochrome-*c* and caspase-9, its expression is lost in the majority of metastatic melanomas (Soengas *et al*., 2001). These observations suggest that attenuation of apoptosis due to the inactivation or silencing of caspases may represent an important mechanism of cell survival and chemoresistance.

Our present findings are in contrast with those of an earlier report by Nakopoulou *et al*. (2001). These authors employed an immunohistochemical assay to study caspase-3 protein expression in breast tumor samples and concluded that 75% of the breast tumors had higher levels of expression of this protein than the non-neoplastic breast tissue. Contrary to these findings, our results suggested that downregulation and deficiency of caspase-3 expression occurs in breast cancer cells and may represent a potential mechanism of cell survival. The observation of high caspase-3 expression by Nakopoulou *et al*. (2001) using immunohistochemical assay can be explained by possible cross reactivity of anti-caspase-3 antibody with some non caspase-3 protein in the breast tissue sections.

Finally, based on our preliminary results using the total RNA isolated from the breast, ovarian, cervical, and uterine carcinomas, it is likely that a similar loss or downregulation of caspase-3 expression may occur in other gynecologic tumors (Figure 3a). At this point, we do not know the mechanisms that may underlie the loss of caspase-3 expression. CpG island hypermethylation, which is known to result in the silencing of several key genes in breast cancer (Huang *et al*., 1999), might play a role. Indeed, silencing of the caspase-8 and caspase-1 genes was recently shown to result from hypermethylation in childhood neuroblastoma and renal cancer cells, respectively (Teitz *et al*., 2000; Ueki *et al*., 2001). It is equally possible that some functional deletions in the *CASP3* gene may result in the loss of its expression. For instance, a 124 bp deletion in the caspase-3 transcript in MCF-7 cells (Jenicke *et al*., 1998) as well as in ZR-75T and BT-20T breast cancer cell lines (unpublished observations) results in a complete loss of caspase-3 protein expression (Figure 1). Moreover, we recently observed that some breast cancer patients harbor a 121 bp deletion ($bp_{483\rightarrow604}$) in the active-site region of the caspase-3 transcript (unpublished observations). A similar deletion in the C-terminal end of the caspase-3 (casp3ΔC) transcript was observed by Huang *et al*. (2001) in a human colon carcinoma cell line; this deletion results from alternative mRNA splicing, and its product acts as a dominant, negative regulator, rendering the cells resistant to apoptosis.

In conclusion, our results suggest that the tumor cells as well as the normal parenchyma surrounding the tumor lack caspase 3 expression in the majority of breast cancer patients. As one would expect, loss of expression or function of this key caspase, can render breast cancer cells resistant to apoptosis in response to certain apoptotic stimuli including chemotherapeutic drugs and thus may affect the outcome and prognosis of the disease. These findings may have important clinical implications in terms of using caspase-3 not only as a marker of disease but also as a therapeutic target for breast cancer.

Materials and methods

Cell lines and tissue samples

The human breast cancer cell lines T47D, MDA-MB-361, MDA-MB-468, BT-474, SKBR-3, BT-20, MDA-MB-157, and MDA-MB-436 and the mammary epithelial cell line, MCF10A were obtained from and maintained according to the instructions provided by the American Type Culture Collection (Rockville, MD, USA). In addition, the MDA-MB-134, Hs854T, HCC1954, HCC1428, DU4475, BT-549, and BT-483 breast cancer cell lines were provided by Dr J Dai (Yuan *et al.*, 2001), and BT-20T and ZR-75T cancer cell lines were provided by Dr K Keyomarsi (Chen *et al.*, 2000). Two normal mammary epithelial (NME) cell lines having a finite life span were purchased from Clonetics (San Diego, CA, USA). The drug-resistant MCF-7/DOX cell line was established via continuous culture of MCF-7 cells in the presence of doxorubicin as described previously (Devarajan *et al.*, 2002). The caspase-3 deficient MCF-7 cells were infected with either pBabe/puromycin retroviral vector alone (pBS) or vector containing full-length caspase-3 cDNA insert (pBS-CPP32), as described previously (Yang *et al.*, 2001).

Primary breast tumor and normal breast parenchyma samples (4–6 mm^3 thick) were obtained from patients who had undergone breast surgery at The University of Texas MD Anderson Cancer Center. A total of 46 patients, including 31 having breast adenocarcinoma (four of whom had received preoperative chemotherapy), 10 with a history of breast cancer, three having benign breast disease, and two undergoing breast reduction surgery were enrolled in this pilot study. The median age of the patients was 55 years. The samples were obtained with the patients' consent following approval by the Institutional Committee for the Welfare of Human Subjects. Microscopic examination of representative sections of each sample after staining with hematoxylin and eosin stain confirmed the presence of carcinoma cells in greater than 80% of the sample area. The samples were either directly collected in RNAzol for immediate isolation of total RNA or snap frozen and stored at −80°C for later use.

Flow cytometric analysis of apoptotic cells

To determine the apoptosis, $1-2\times10^6$ cells were seeded into T-75 tissue culture flasks the day before treatment with 2 μM ionophore A23187 or 50 nM staurosporine (SSP) (both from Sigma). After 48 h treatment, free-floating cells were removed from the flasks and pooled with adherent cells obtained by trypsinization from the respective flasks. Pooled cells were permeabilized and incubated with 8 μg/ml acridine orange (Polysciences, Warrington, PA, USA). Cell fluorescence was measured using the logarithmic scale of the FACScan flow cytometer with a 488 nm excitation of a 15 mW argon laser and filter settings for green (530 nm; DNA) and red (585 nm; RNA) fluorescence. Ten thousand events were stored and run for analysis following the elimination of cell debris and doublets. The percentage of cells in the 'sub G1' region defined the proportion of apoptotic cells in the tested populations.

Reverse transcriptase-polymerase chain reaction (RT–PCR) analysis

RT–PCR analysis of caspase-3 transcript was carried out as described previously (Devarajan *et al.*, 2002). Briefly, total RNA was isolated from tissue samples or cells using the TRIzol method (Guo and Kyprianou, 1999). RT–PCR was performed using 5 μg of total RNA and the Superscript II reverse transcript kit (Life Technologies, Grand Island, NY, USA) in an amplification cycler (Perkin-Elmer, Wellesley, MA, USA). The sequences of the caspase-3-specific primers used were as follows: sense, 5′-TTAATAAAGGTATC-CATGGAGAACACT-3′; and antisense, 5′-TTAGTGATA-AAAA TAGAGTTCTTTTGTGAG-3′. RT–PCR was performed at 94°C for 2 min, 33 cycles at 94°C for 1 min, at 55°C for 1 min, and at 72°C for 5 min. The integrity of the RNA used for RT–PCR was confirmed using β-actin synthesis as a positive control reaction (Haidar *et al.*, 2000). The amplified RT–PCR products were separated on a 1% agarose gel, stained with ethidium bromide for visualization, and photographed under ultraviolet illumination. For complementary DNA (cDNA) sequencing, the PCR products were subcloned into pCR2.1 plasmid (Invitrogen, Carlsbad, CA, USA) and sequenced in both directions using an automated DNA sequencer (310 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA).

Northern blot analysis

Total RNA was isolated from primary breast tumor samples as described above. Also, to study caspases-3 expression in other gynecological tumors, total RNA isolated from human breast, cervical, ovarian, and uterine tumor tissues was purchased from Clontech Laboratories (Palo Alto, CA, USA). Ten micrograms of total RNA was mixed with the sample buffer (2.2 M formaldehyde, 50% formamide, 50 mM MOPS (pH 7.0) and 1 mM EDTA), denatured via heating at 65°C for 10 min, resolved on 1.5% agarose/formaldehyde gels, and transferred to a Hybond nylon membrane. In addition, nylon membrane containing messenger RNA (mRNA) isolated from various normal human tissues was purchased from Clontech Laboratories (Multiple Tissue Northern (MTN™) blot). Caspase-3-specific probe of 824 base pairs (bp) was generated using human mammary epithelial cDNA and caspase-3-specific primers. The PCR product of caspase-3 was oligolabeled with ^{32}P-α-dCTP by random labeling (Boehringer Mannheim Corp., Indianapolis, IN, USA). Hybridizations were carried out overnight at 42°C using random primed labeled caspase-3 probe in Quikhyb (Stratgene, La Jolla, CA, USA) in accordance with the manufacturer's instructions. Furthermore, filters were exposed to autoradiographic film for up to 5 days. To test for the uniform loading of the samples, blots were stripped and reprobed using a cDNA probe for the human 18S rRNA or GAPDH.

The caspase 3 transcript in few selected samples was also determined by using an adaptation of RT–PCR *in situ* (RT–PCR IS) as described by Bagasara *et al.* (1993). The 5′-TTAATAAAGGTATCCATGGAGAACACT-3′ primer was labeled with digoxigenin (Sigma Genosys, The Woodland, TX, USA) and PCR was performed for 35 cycles at 94°C for 50 s, at 55°C for 30 s, and at 72°C for 1 min.

Western blot analysis

PBS-washed cells were lysed by sonication in ice-cold buffer A (20 mM Tris-buffered saline (pH 7.4) containing 1 mM EDTA, 14 mM 2-mercaptoethanol, and 1 μM phenylmethyl-sulfonyl fluoride). Lysed cells were centrifuged at 14 000 r.p.m. for 10 min to remove cell-debris. Protein extracts from breast cancer tissue samples were prepared by either directly grinding the tissue samples using a prechilled mortar and pestle in buffer A or extensively dialyzing the tissue extracts left behind after the total RNA extraction

against phosphate-buffered saline containing 0.1% sodium dodecyl sulfate (SDS) (Chomczynski, 1993). The dialyzed extracts were then concentrated using Centricon cartridge filter (10 kDa cut-off; Amicon Bioseparations, Bedford, MA, USA), and the protein content in cell and tissue extracts was determined using BioRad dye reagent I (Richmond, CA, USA). Equal amounts of protein (30–60 μg) were subjected to 12% SDS-polyacrylamide gel electrophoresis (PAGE), and the proteins were transferred onto nitrocellulose membranes. After overnight blocking with 5% nonfat dry milk, the membranes were probed with an anti-caspase-3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:2000, or anti-caspase-8 or anti-caspase-9 antibody (Cell Signaling Technology, Beverly, MA, USA) at a dilution of 1:2000. All of the membranes were stripped and reprobed with an anti-β-actin antibody (Sigma Chemical Co, St. Louis, MO, USA) at a dilution of 1:2000. The Amersham Pharmacia Biotech ECL system (Piscutaway, NJ, USA) was used to detect the antigen-antibody reaction.

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